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Edited by

L. FARKAS, M. GÁBOR and F. KÁLLAY

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PREFACE

As a new link in the chain of Hungarian Bioflavonoid Symposia, the 7th meeting was held in Szeged, Hungary, May 16-18, 1985. The aim of the Hungarian Bioflavonoid Symposia has traditionally been to establish an international forum for the exchange of ideas for scientists covering, in the widest possible sense, the special fields involving flavonoid research: chemistry, biochemistry, phytochemistry, plant physiology and taxonomy, pharmacology, and clinical practice. The lectures are never divided into sections, a principal aim of the symposia being to allow the participants also to become acquainted with the recent work and latest results obtained with flavonoids in fields other than their own. This interdisciplinary approach is believed to be highly stimulating.

The present meeting was attended by participants from 15 countries. The valuable contributions of 44 lectures covering different fields, yet all dealing with flavonoids or closely related compounds, are included in this book. The editors feel that the several newly discovered biological actions of flavonoid compounds, and the elucidation of the mechanisms of these actions by modern methods, might be of particular interest to the reader. Such readers may be engaged either in isolating, synthesising, or investigating the mode of action of flavonoids, or employing them as new medicinal agents. We hope this volume provides new information that will be found useful and interesting by chemists, botanists, pharmacologists, medical doctors and all those whose work is in any way concerned with the research and uses of flavonoid compounds.

It is the Editors' pleasant duty to acknowledge with gratitude the financial support of several pharmaceutical and chemical firms in Hungary, which materially assisted the organization of the 7th Hungarian Bioflavonoid Symposium.

The Editors

CONTENTS

Preface v

List of contributors xi

M. GÁBOR
Opening address xix

L. FARKAS
Address of welcome xxi

D.M.X. DONNELLY and P.G.M. CANNON
The synthesis and biological activity of the latinones 1

M. NÓGRÁDI, S. ANTUS, Á. GOTTSEGEN, D. KORBONITS, J. SZEJTLI, Á. SZŐKE, ZS. FÜRST and J. KNOLL
Synthesis and analgesic properties of some cannabionids with ether type side chains 15

S. ANTUS, R. BAUER, Á. GOTTSEGEN, O. SELIGMANN and H. WAGNER
Synthesis of americanin-D 31

M. RÁKOSI, ZS. SZEGÉNY, J. BÁLINT and R. BOGNÁR
Synthesis of some novel flavonoid derivatives 39

A. LÉVAI, G. TÓTH and A. SZŐLLŐSY
Synthesis of benzopyrano[4,3-c]pyrazolines and related compounds 47

C.S.R. IYER and P.R. IYER
Synthesis of natural pyranochromones 55

B. VERMES and H. WAGNER
Natural xanthones and their glycosides 63

E. PÉLI, T. PATONAY, M. ZSUGA and GY. LITKEI
Kinetic investigations of the elimination reaction of 3-bromoflavanones 75

T. PATONAY and GY. LITKEI
Reaction of 3-mesyloxyflavanones with S-nucleophiles 85

Z. DINYA, S. KISS, J. BÁLINT and R. BOGNÁR
Quantum chemical studies on the UV spectra of parent flavonoids 93

D. REHN and G. WURM
Ethylene carbonate, a regioselective reactant for hydroxyethylation of flavonoids 103

E. HASLAM and T.H. LILLEY
Polyphenol complexation 113

H. WAGNER, O. SELIGMANN and H. LOTTER
Recent advances in the chemistry, stereochemistry, synthesis and pharmacology of flavanoligans 139

E. WOLLENWEBER
Flavonoid aglycones as leaf exudate constituents in higher plants 155

Á. KÉRY, H.A. TWAIJ and A.A. AL-JEBOORY
Flavonoid composition in some Iraqi plants used in folk medicine 171

J.W. McCLURE, D.H. NETZLY and E.G. COSIO
Tissue-specific production of flavonoids in *Hordeum* and in *Glycine* 183

E. FIEDLER, C.L. SCHMIDT, C. GROβ and G. SCHULTZ
Chloroplasts of higher plants are the site of synthesis of primary aromatic compounds: compartmentation and partial characterization of crucial enzymes of the shikimate pathway 195

K.M. VALANT-VETSCHERA and E. WOLLENWEBER
External accumulation of flavonoid aglycones in *Achillea* 213

É. NAGY, V. PÁPAY, GY. LITKEI and Z. DINYA
Investigation of the chemical constituents, particularly the flavonoid components, of propolis and *Populi gemma* by the GC/MS method 223

V. PÁPAY, L. TÓTH, M. SOLTÉSZ, É. NAGY and GY. LITKEI
Isolated compounds from Hungarian propolis and *Populi gemma* 233

G. VERZÁR-PETRI, G. MARCZAL and P. OROSZLÁN
Polyphenols of *Cotinus coggygria* and their therapeutical value 241

N. NIKOLOV, O. SELIGMANN and H. WAGNER
New flavonol glycosides from *Mespilus germanica* L. 251

GY. SULYOK and J. BÁLINT
Identification of the anthocyanin of *Consolida orientalis* Schröd 261

É. NAGY, A. NESZMÉLYI and G. VERZÁR-PETRI
Characteristic flavonoids of *Lilium candidum* L. and their distribution in different plant parts 265

E. GÁBOR and O. HERCZEGH
Isolation of anthocyanidins from natural sources 273

M. GELLÉRT, K. SZENDREI, Z. DINYA and J. RÉPÁSI
Characteristic constituents of the cherry peduncle extract Novicardin: HPLC fingerprint 279

C. VILAIN, J. DAMAS, J. LECOMTE and L.Y. FOO
Pharmacological properties of a novel flavan derivative isolated from the New Zealand conifer *Phyllocladus alpinus:* cardiovascular activities of phylloflavan in rats 287

R. BONIFACE, M. MISKULIN, L. ROBERT and A.M. ROBERT
Pharmacological properties of *Myrtillus* anthocyanosides: correlation with results of treatment of diabetic microangiopathy 293

M. GÁBOR
New results in the pharmacology of benzopyrone derivatives and related compounds 303

A.L. KAZAKOV, V.P. KHILYA and V.A. KOMPANTSEV
Isoflavones and arteriosclerosis 321

I. BÉLÁDI, Y. MÁNDI, GY. SEPRÉNYI, R. PUSZTAI and A. VECKENSTEDT
The effect of flavonoids on the natural killer activity of human lymphocytes 325

I. MUCSI
Combined antiviral effect of quercetin and interferon on the multiplication of herpes simplex virus in cell cultures 333

A. VECKENSTEDT, J. GÜTTNER, R. PUSZTAI, H. HEINECKE, A. HÄRTL and I. BÉLÁDI
Antiviral, immunologic and toxicologic studies with quercetin in mice 339

K. DRIEU, J.-P. MOREAU, C.-R. ECK, J. McCABE and S. SKINNER
Animal distribution and preliminary human kinetic studies of the flavonoid fraction of a standardized *Ginkgo biloba* extract (GBE 761) 351

J. SONNENBICHLER and I. ZETL
Influence of the flavanolignan derivative Silibinin on nucleic acid and protein synthesis in liver cells 361

J.-P. CAZENAVE, A. BERETZ and R. ANTON
The effects of flavonoids on human platelet function 373

E. MIDDLETON, Jr.
Some effects of flavonoids on mammalian cell systems 381

F. MICHEL, L. MERCKLEIN, R. REY and A. CRASTES de PAULET
Comparative effects of some flavonoids on cyclooxigenase and lipoxygenase activities in different cell systems or subfractions 389

J. BAUMANN, I. JUST and G. WURM
Oxidation of suitable drugs by horseradish peroxidase/hydrogen peroxide. Results in the oxygenation of arachidonic acid to form hydroperoxyeicosatetraenoic acids in a reverse cooxidation process 403

J. KÖHRLE, M. AUF'MKOLK, M. SPANKA, G. SÓNYI, V. CODY and R.D. HESCH
Flavonoids inhibit enzymic thyroid hormone deiodination 411

J. PINCEMAIL, C. DEBY, Y. LION, P. BRAQUET, P. HANS, K. DRIEU and R. GOUTIER
Role of flavonoids in lipoperoxidation and radicalar reactions 423

L.A. GRIFFITHS, A.M. HACKETT and I.C. SHAW
Altered patterns of drug metabolism in liver disease and their normalization by cyanidanol-3 437

I. ALTORJAY, L. DALMI and B. SÁRI
Investigation of the protective effect of (+)-cyanidanol-3 against paracetamol-induced membrane damage in erythrocytes 445

G.A. BÁLINT, M. GÁBOR, J. NÁFRÁDI and V. VARRÓ
Interaction of 3-O-methyl-(+)-catechin and H_2-receptor blockers in piroxicam- and indomethacin-induced gastric ulcer models of rat 453

Subject index 457

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OPENING ADDRESS

by

PROF. DR. M. GÁBOR

Ladies and Gentlemen,

The Seventh Hungarian Bioflavonoid Symposium has been organized to commemorate the 50th anniversary of the discovery by Rusznyák and Szent-Györgyi in Szeged of the therapeutical effects of flavonoids.

In opening this Symposium, I think it is appropriate to try to give you a brief historical survey of flavonoid research in Hungary.

In 1935 Szent-Györgyi isolated a substance from lemon juice which decreased the permeability and increased the resistance of the capillary wall. Chemical analysis indicated that this substance was a flavonoid and it was named "citrin". Bruckner and Szent-Györgyi (1937) found that "citrin" was composed of hesperidin and eriodictiol glycoside. Huszák (1937) reported on the fate of parenterally administered "citrin".

A significant part in the research in Hungary was played by Jeney, who in 1936 reported the favourable effects of flavonoids on intact and poisoned isolated frog heart. Other internationally recognized experts in flavonoid chemistry included Zemplén, Bognár, Farkas and their schools.

The discovery in Szeged called attention to the biological action of flavonoids, and from 1940 onwards many papers were published by foreign researchers on the effects of flavonoid compounds, such as catechins, leucoanthocyanidins (procyanidins), tea catechis, rutin, etc.

Twenty years after Rusznyák and Szent-Györgyi's discovery the first flavonoid conference was organized in the United States under the name "Bioflavonoids and the Capillary", where Szent-Györgyi gave a lecture entitled "Perspectives for the Bioflavonoids".

I should mention that the Working Committee for Flavonoid Research of the Hungarian Academy of Sciences organized the First Hungarian Bioflavonoid Symposium in Szeged in 1965. Our international symposia are now held every 4 years; the previous one was organized by Professor Wagner in Munich.

Numerous congresses and conferences have been devoted to the flavonoids in Europe during the past twenty years, all showing the growing interest in these compounds. In two months' time, a symposium "Plant Flavonoids in Biology and Medicine" will be held in Buffalo.

Fifty years after the pioneering work by Rusznyák and Szent-Györgyi, the question naturally arises as to the explanation of the renaissance in flavonoid research. The answer is clear: chemists have synthetized new flavonoid derivatives with previously unknown biological effects; phytochemists have isolated numerous new flavonoids from many plants; biochemists have demonstrated the most varied effects on different enzymes; and an ever increasing number of pharmacologic effects have become known through variations of the chemical structures of the flavonoids and related compounds. This research has led to the discovery of many new drugs that can be applied for therapeutic purposes.

Hungarian flavonoid researchers started the useful work on these interesting natural, semisynthetic, or synthetic compounds here in Szeged, and I am sure that this present conference will provide us with accounts of many new results, and will give ample possibilities for the mutual exchange of experience. Let me wish every success to all the participants in this meeting.

I declare the Symposium open.

ADDRESS OF WELCOME

by

PROF. DR. L. FARKAS

Ladies and Gentlemen,

I have the great pleasure of welcoming you to this symposium on behalf of the Working Committee for Flavonoid Research of the Hungarian Academy of Sciences.

In his opening address Professor Gábor told us about our great traditions and the pioneering work of Hungarian scientists in this field. Our Working Committee for Flavonoid Research was established twenty years ago to facilitate and support the further contribution of Hungarian researchers to the chemistry, biochemistry and all other scientific or practical aspects of this interesting group of natural compounds. Moreover, an important aim of the Committee has been to ensure that our work in Hungary remains correctly integrated with that of others in the rest of the world.

This Seventh Hungarian Bioflavonoid Symposium seems to confirm again that our efforts have not been in vain. Just the fact that you all have honoured this meeting by coming to Szeged from distant countries and from various parts of Hungary is a sure mark of your appreciation, which makes us - the organizers of the present and former Symposia - proud and happy.

It is your presence and contributions which will certainly make this Symposium meaningful. We are convinced that the free exchange of our experience and ideas, discussions about the latest results and most promising trends of future research, and the possibility of doing so in person for three or four days, will be rewarding and enjoyable.

In this spirit, wishing you a useful and pleasant stay in this characteristically Hungarian town, Szeged, I extend a cordial welcome to you.

Flavonoids and Bioflavonoids, 1985
(Eds. L. Farkas, M. Gábor, F. Kállay)

THE SYNTHESIS AND BIOLOGICAL ACTIVITY OF THE LATINONES

DERVILLA M.X. DONNELLY and PETER G.M. CANNON

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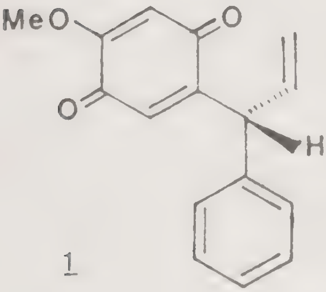
INTRODUCTION

The irritant effect of rosewoods was attributed to the presence in the heartwood of flavones, saponins and glycosides but the discovery of sensitising quinones in other timbers led to a search for similar substances in the Dalbergieae /1/. Some thirty species of *Dalbergia* and *Machaerium* have been investigated and quinones with neoflavanoid and isoflavonoid skeletal structures were found. The quinones of the neoflavanoid group have open chains and the most commonly occurring are R- and S-4-methoxydalbergione (1) and (2) /2/.



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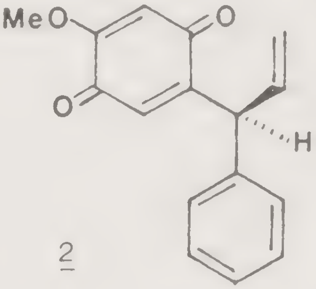

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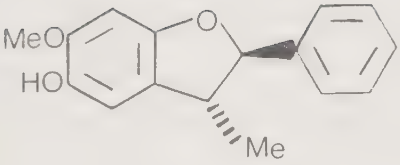
The quinone which may arise from ring opening of obtusafuran (3) can be considered an isoflavonoid (C_6-C_2-C_6) and it occurs as one moiety of dalcriodain (4) /3/.



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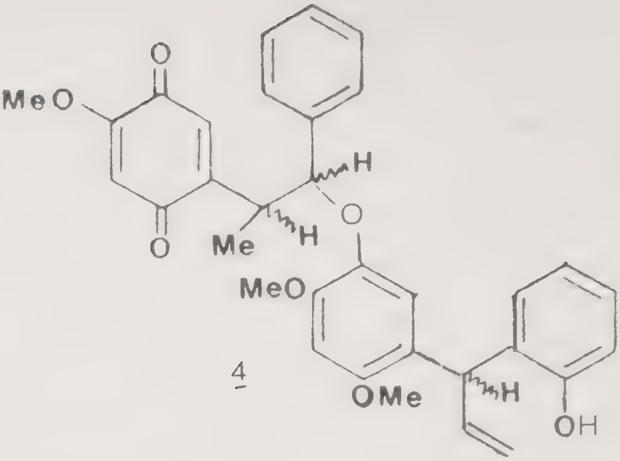

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A third quinone structure a phenanthra - 1,4- quinone, called latinone (5) /4/ was found in four species of Dalbergia (D. latifolia, Roxb. D. baroni, Baker D. cochinchinensis Pierre and D. melanoxylon Guill et Perr.). A second example of this group mellatinone (6) was present in D. melanoxylon /5/. The occurrence of compounds such as 4-methoxydalbergiones, 4-phenylcoumarins and benzophenones in the same species as the similarily oxygenated phenanthra - 1,4 - quinones 5 and 6 suggests that the latter compounds may be biogenetically related to the neoflavanoids. Table 1, which summaries the neoflavanoids found in D. melanoxylon underlines these structural similarities. The co-occurrence of mellanoin, S-4'-hydroxy-4-methoxydalbergione and melanettin points to the possible presence in D. melanoxylon of a third phenanthra - 1,4 - quinone bearing a 9-(4'-hydroxyphenyl) substituent. This quinone has not been isolated to date, due probably to the lower concentration of this series compared with the other neoflavanoids in the wood.

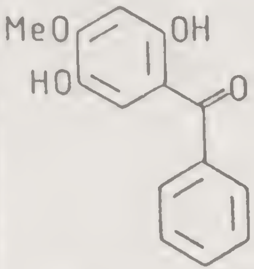
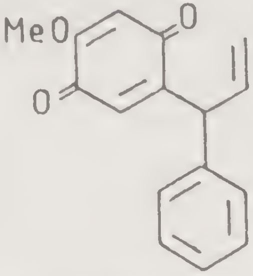
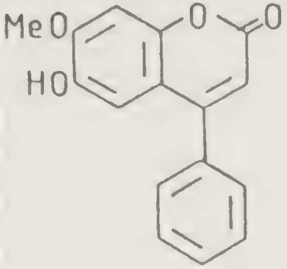
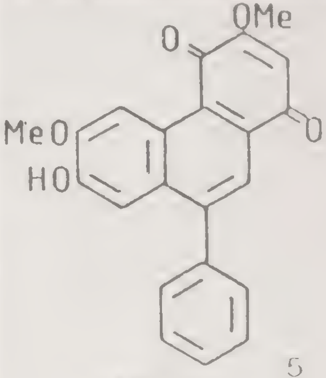
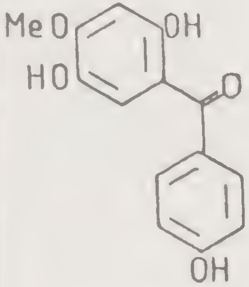
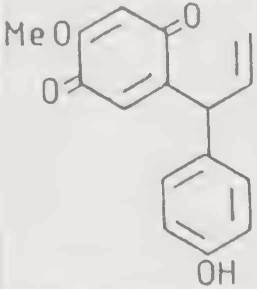
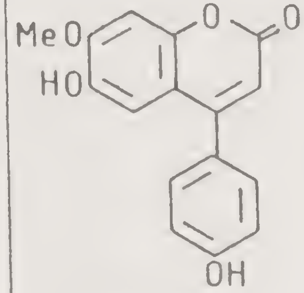
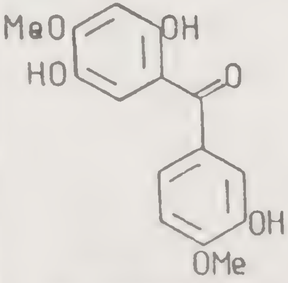
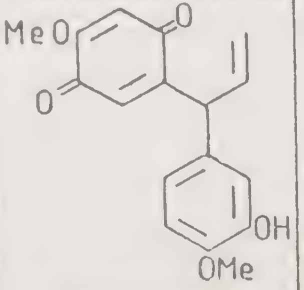
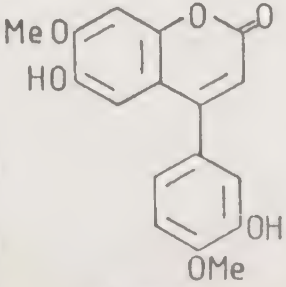
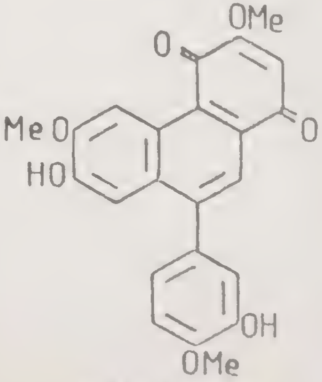
By analogy to the biosynthetic proposals for the neoflavanoids of the Leguminoseae, it has been suggested that the $C_6-C_2-C_6$ unit of the phenanthraquinones may arise by cinnamylation of a phenol followed by loss of a carboxyl group and of an oxygen, a loss which occurs probably at the condensation step with an additional C_6 unit

DISCUSSION

Structure of Latinone (5)

The structure of the natural product latinone (5) was assigned initially on the basis of its spectroscopic properties and subsequently on chemical

Table 1 Neoflavanoids of *Dalbergia melanoxylon* Guill et Perr.

	Benzophenone	Dalbergione	4-Phenylcoumarin	9-Phenyl-1,4-phenanthraquinone
no substitution in B-ring	 cearoin	 (S)-4-methoxy-dalbergione	 dalbergin	 latinone 5
4-hydroxy substituent in B-ring	 melannoin	 (S)-4-methoxy-4'-hydroxydalbergione	 melanettin	—
3-hydroxy-4-methoxy-substituent in B-ring	 melanoxoin	 melannone	 melannein	 mellatinone 6

evidence. The position of the methoxy group on the quinone ring was located when 7-O-methyllatinone (7) was hydrolysed and the 2-(or 3)-hydroxyquinone obtained was reduced and immediately treated with boric acid. The free hydroxyl in the borate complex was methylated. The complex was then hydrolysed and the product oxidised to afford a mixture of o-quinone and p-quinone. In the o-quinone the change in the environment of the C-10 proton was evident in its ^1H nmr spectrum with an upfield shift of the signal. These results have been reported /4/. Recently the ^{13}C nmr spectrum of 7-O-methyllatinone (7) was obtained and was found to be consistent with the assigned structure. The spectrum (relevant assignments summarised in Figure 1) had two characteristic singlets at δ185.51 and 182.66 which were assigned to C-1 and C-4 respectively. The downfield shift at C-4, relative to the unsubstituted analogue, is attributed to the -OMe group at C-3. The signals for C-2, C-5 and C-8 (δ105.29, δ106.22 and δ107.32) are reversible, whilst C-10 registered a doublet at δ122.17. The quaternary C-9 was assigned to the signal δ145.97.

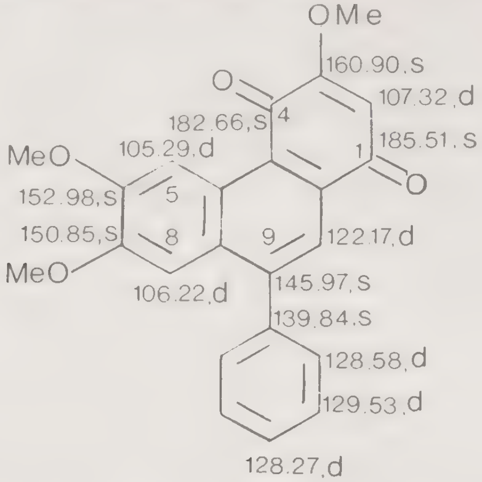
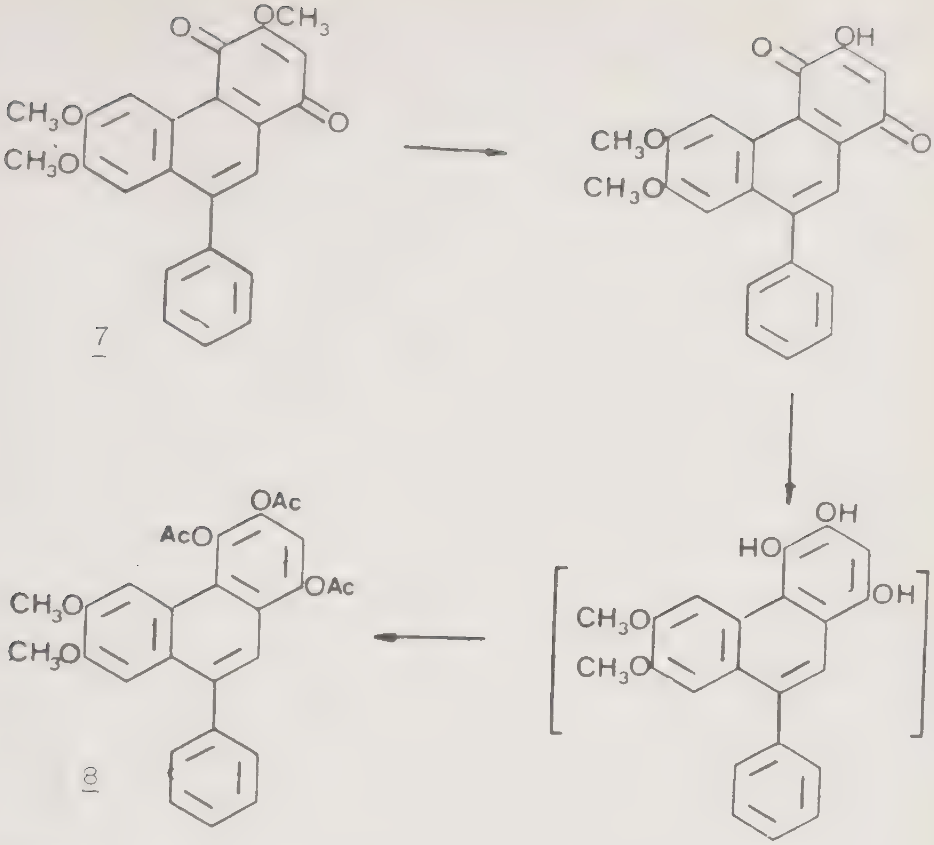


Figure 1

Final confirmation for the structure of latinone (5) came from a single crystal X-ray analysis of 1,3,4-triacetoxy-6,7-dimethoxy-9-phenylphenanthrene (8). The triacetate (8) was prepared by two routes: (i) selective demethylation of latinone (5) followed by a reductive acetylation (Scheme 1), and (ii) via a Thiele acetylation of 6,7-dimethoxyphenanthra-1,4-quinone. The molecular structure of the triacetate 8 is shown in Figure 2.

Latinone (5) resembles the sensitising phenanthra-1,4-quinone cypripedin (7-hydroxy-2,8-dimethoxyphenanthra-1,4-quinone) which was isolated



Scheme 1

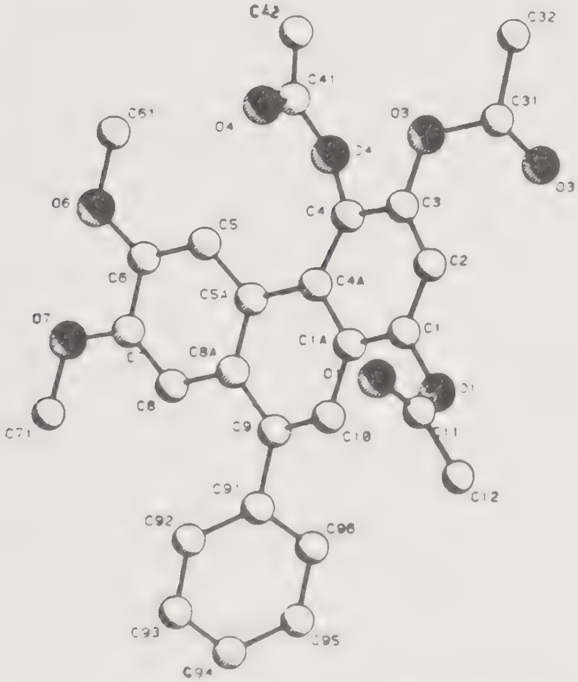


Figure 2. Molecular structure of 1,3,4-triacetoxy-6,7-dimethoxy-9-phenylphenanthrene.

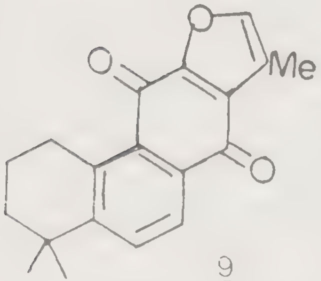
from the orchid *Cypripedium calceolus L.* (Lady Slipper) /6/. The quantity of latinone (5) and mellatinone (6) present in the heartwood is exceedingly small (≈ .0005%) and was insufficient for biological testing. Two cases of allergic contact dermititis due to repeated contact with a *Dalbergia* species, have been recorded. In the first case only one quinone was positive and was observed not to be a dalbergione whilst R- and S-4-methoxydalbergione (1) and (2), R-3,4-dimethoxydalbergione and S-4,4'-dimethoxydalbergione elicited positive skin responses in the second case /1/.

With a view to investigating structure-activity relationships the synthesis of latinone and its analogues was undertaken. The notable structure differences between the active cypripedin and the phenanthra-1,4-quinones of the rosewoods were the position of the methoxyl group in the quinone ring and presence of the aryl group on C-9.

Synthesis of 7-O-methyllatinone (7)

The Diels-Alder reaction has many applications in elegant syntheses of natural products among which the tanshinones and isotanshinone ll (9) number. In the current study, the Diels-Alder reaction was used to build the basic synthon of latinone, mellatinone and related compounds.

In using this reaction for formation of the phenanthraquinone 5, the problem of the orientation in the Diels-Alder adduct due to unsymmetrical benzoquinones exists and would be similar to that encountered in the synthesis of isotanshinone ll (9) /7/. The effect of substituents in the condensation may be considered from two points: (i) radical stability in an



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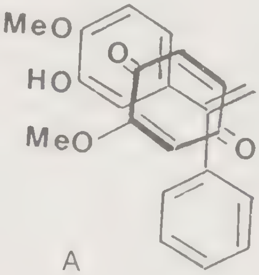
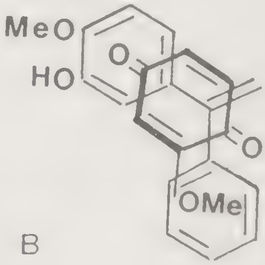


A



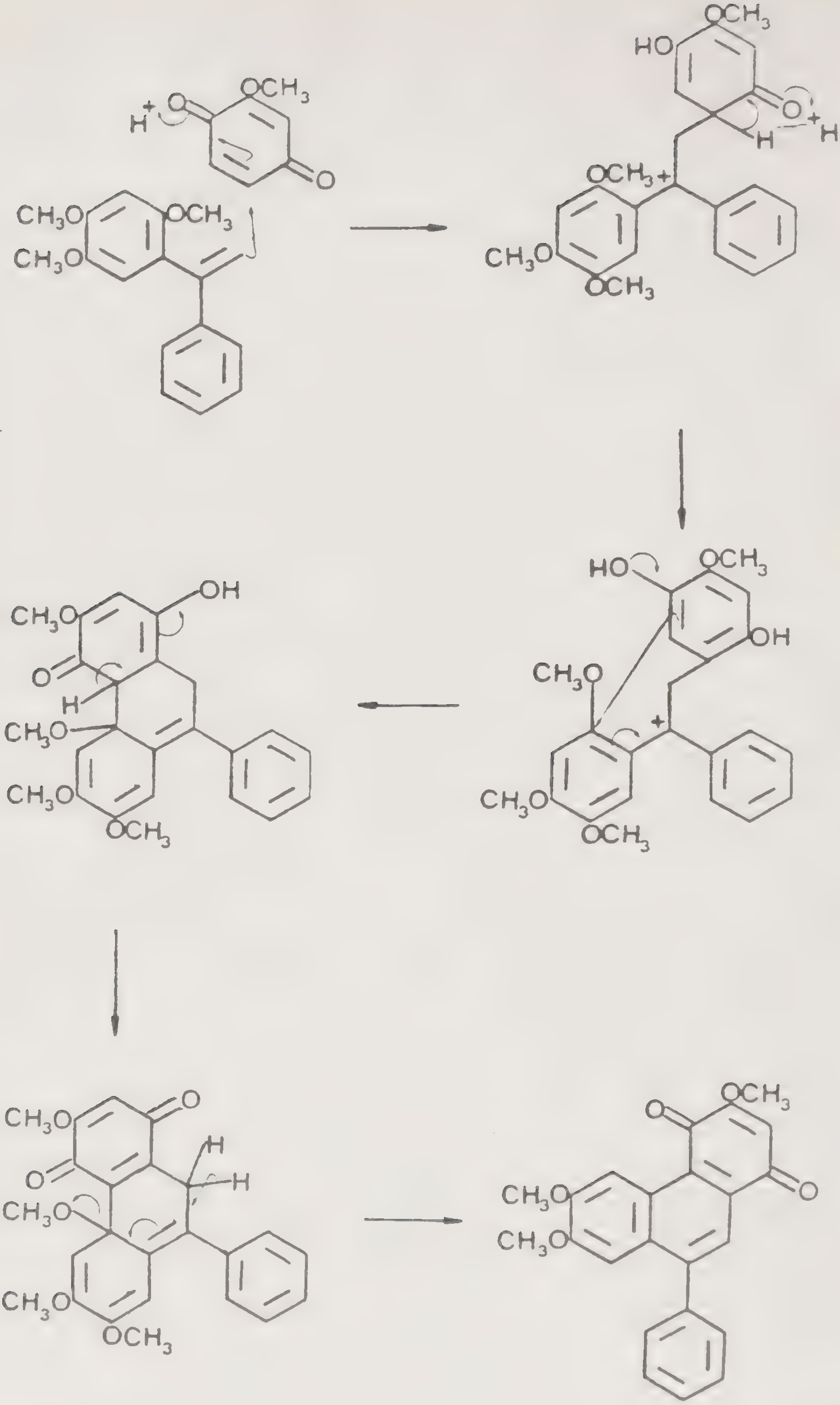
B

assumed biradical intermediate and (ii) the steric factor in the transition state. In the formation of latinone-7-O methyl ether from the reaction of 1-(3,4-dimethoxyphenyl)-1-phenylethylene with 2-methoxy-1,4-benzoquinone, the transition state A would be energetically more favoured than B in which the bulky OMe and Ph groups overlap. As predicted the product of the reaction proved to be 3,6,7-trimethoxy-9-phenylphenanthra-1,4-quinone (7-O-methyllatinone).

Methylation of cearoin, one of the benzophenones present in abundance in Dalbergia species, afforded a trimethyl ether which was converted to 1-(2,4,5-trimethoxyphenyl)-1-phenylethylene via a Grignard or Wittig reaction. In this "diene" the reaction site leading to the formation of latinone derivatives is blocked by a methoxyl group. In the "Diels-Alder" reaction (with 2-methoxy-1,4-benzoquinone) in acetic acid at 116°C 7-O-methyllatinone was obtained as the minor product. This result involves the loss of an angular methoxyl group from the adduct. A mechanism to account for this reaction is presented in Scheme 2. The loss of the H^+ ions could be satisfied in the uptake by the excess benzoquinone found essential for the reaction to take place. The second and the major product formed in the reaction was 5-hydroxy-6-methoxy-3-(2',4',5'-trimethoxyphenyl)-2-phenylbenzo[b]-furan (10). In the EIMS of the benzo[b]furan the molecular ion and base peak were at m/z 406. Only one peak in the spectrum m/z 391 (M^+-CH_3) had an intensity greater than 20% of the base peak. The spectrum exhibited the characteristic $[M-C_6H_5CO]^+$ signal at m/z 301 and $[M-CHO]^+$ at m/z 377. The ^1H nmr spectrum registered the aromatic signals and four methoxyl singlets (δ3.66, 3.79, 3.95 and 4.02). The signal at δ5.61 (exchangeable with D_2O) was assigned to the C-5 hydroxyl. The structure 10 was confirmed by single crystal X-ray analysis of the 5-acetoxy derivative (Figure 3).

Initially it was considered that the benzo[b]furan 10 may have arisen via a stilbene intermediate. It was possible that the 1-(2,4,5-trimethoxyphenyl)-1-phenylethylene had undergone a Wagner-Meerwein type rearrangement in the polar solvent to form the corresponding E-stilbene /8/. Attempts to carry out this rearrangement failed.

A mechanism for the formation of the benzo[b]furan (10) is given in Scheme 3. The oxonium ion proposed (Scheme 2) in the formation of the 7-O-methyllatinone (7) is the common intermediate. But whereas in the formation of the phenanthra-1,4-quinone attack occurs at the 2-methoxyl position of the diphenylethylene moiety, for the formation of the benzo-



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Scheme 2

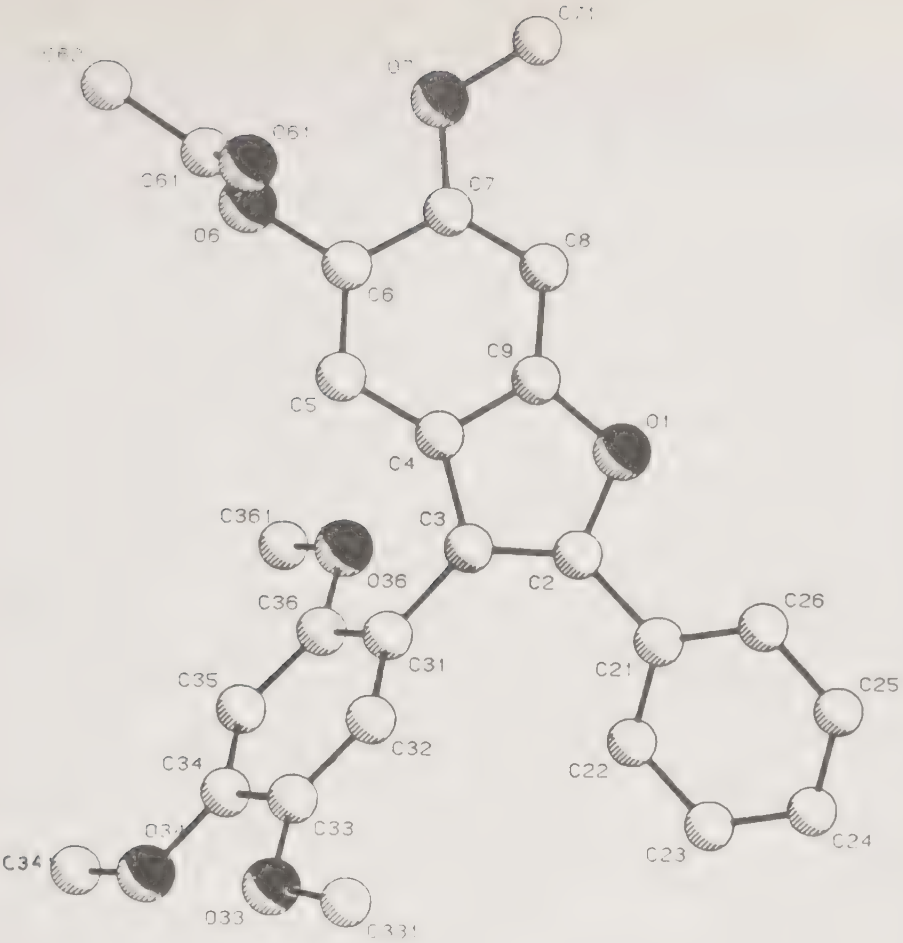


Figure 3. Molecular structure of 5-acetoxy-6-methoxy-3-(2',4',5'-trimethoxyphenyl)-2-phenylbenzo[b]furan.

[b]furan (10). cyclisation occurs to one of the oxygen functions of the quinone. This mechanism requires the acetate ion from the solvent (CH_3COOH) to act as a nucleophile and to participate as the driving force for the 1,2-phenyl migration, crucial in the formation of the carbocation leading to the final product. This mechanism requires the loss of a hydride ion assumed to be accepted by the excess benzoquinone.

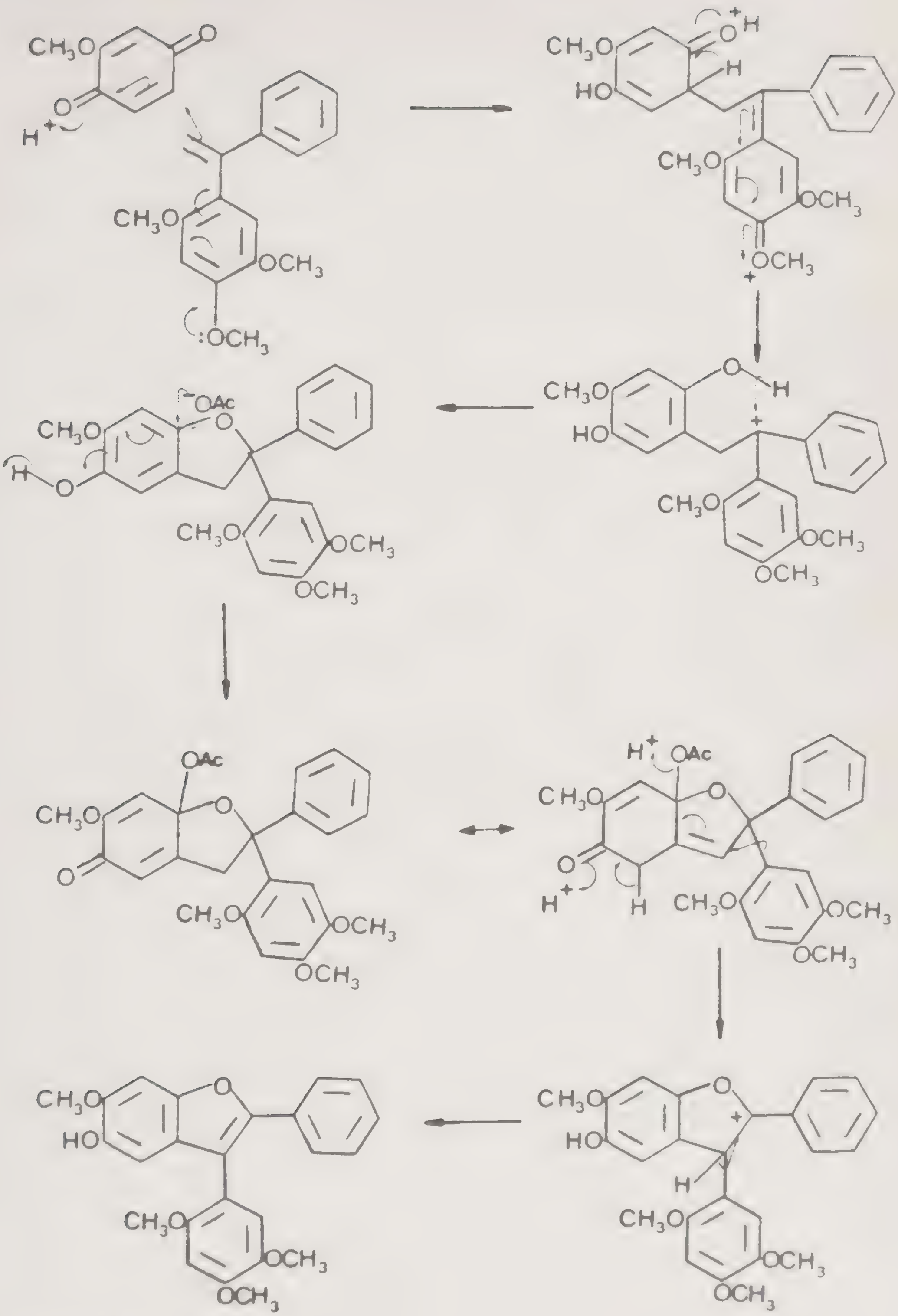
Synthesis of substituted phenanthra-1,4-quinones.

The loss of the 2-methoxyl group was observed also when 1-(2,4,5-trimethoxyphenyl)-1-phenylethylene was reacted in acetic acid at 116° with other benzoquinones. In the presence of 1,4-benzoquinone two products were identified: 6,7-dimethoxy-9-phenylphenanthra-1,4-quinone and 5-hydroxy-3-(2',4',5'-trimethoxyphenyl)-2-phenylbenzo[b]furan, whilst with 2-methyl-1,4-benzoquinone three products were identified: 6,7-dimethoxy-2 (and 3)-methyl-9-phenylphenanthra-1,4-quinones and 5-hydroxy-7-methyl-3-(2',4',5'-trimethoxyphenyl)-2-phenylbenzo[b]furan. Whilst a substituent in the 2-position of the diphenylethylene favours benzo[b]furan formation, it is not essential for its formation. The reaction of 1-(3,4-dimethoxyphenyl)-1-phenylethylene and 1,4-benzoquinone yielded the corresponding benzo[b]furan.

Synthesis of 6,7-dimethoxy-10-methyl-9-phenylphenanthra-1,4-quinone (11)

In adopting a Diels-Alder reaction for the synthesis of the phenanthraquinone (11), it was necessary to prepare 1-(3,4-dimethoxyphenyl)-1-phenylprop-1-ene (an "isoneoflavanoid"). The condensation reaction in xylene with a catalytic amount of CCl_3COOH gave the best yields. The ^1H nmr, EIMS, uv, ir spectra of the product were consistent with the structure of 11.

The major degradation product (30%) isolated from the reaction was 3,4-dimethoxybenzophenone. The possibility that the benzophenone was present as an impurity in the diphenylprop-1-ene starting material was ruled out. No benzo[b]furan was isolated from this reaction. This observation is of interest in the light of the mechanism proposed for the formation of the benzo[b]furan (Scheme 3). In this mechanism a 1,2-phenyl migration is proposed to occur in the conversion of the 2,3-dihydro-2,2-diphenylbenzo-[b]furan intermediate to form the 2,3-diphenylbenzo[b]furan. The $CH_3CO_2^-$ anion from the solvent was considered the driving force behind the migration. The presence of the 3-methyl substituent on the intermediate furan ring



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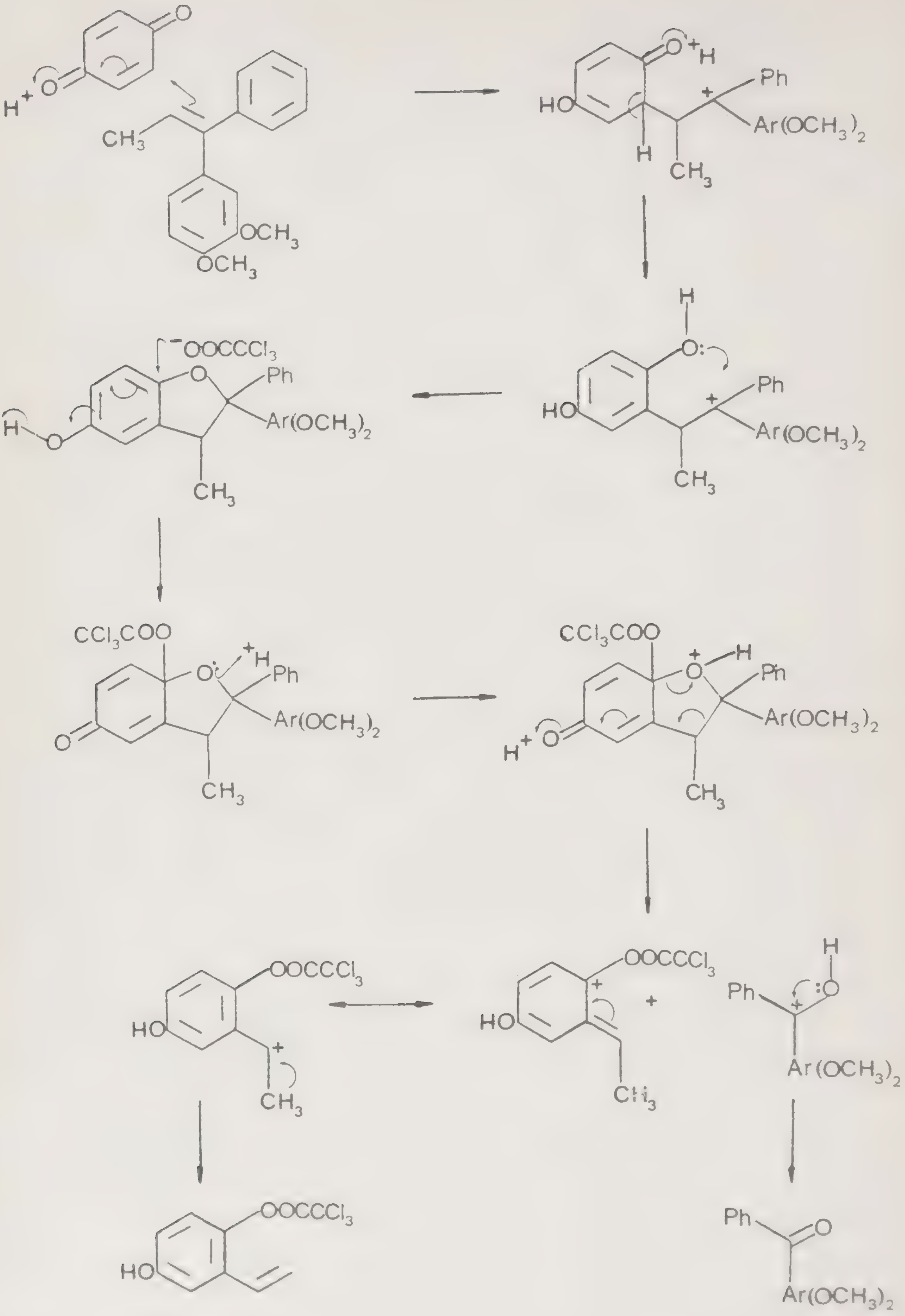
Scheme 3



Scheme 4

precludes the possibility of migration occurring (Scheme 4).

Biological activity of phenanthra-1,4-quinones

It has been shown /9/ that the quinones primin, R-3,4-dimethoxydalbergione, deoxylapachol and cypripedin are potent naturally occurring contact allergens. The common factor in the first three quinones is the presence of a methoxyl group at C-2. Some synthetic 7,8-substituted phenanthrene-1,4-quinones as well as the natural cypripedin show high sensitising capacity probably due to the out-of-plane position of the C-8 methoxyl group. Neither latinone nor mellatinone showed activity. In both these natural products the C-2 and C-8 positions are unsubstituted.

Many of the phenanthra-1,4-quinones discussed excluding 7-O-methyllatinone (7) showed some *in vitro* activity against yeasts (Candida), dermatophylic fungi and trichomonads.

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Flavonoids and Bioflavonoids, 1985
(Eds. L. Farkas, M. Gábor, F. Kállay)

SYNTHESIS AND ANALGESIC PROPERTIES OF SOME CANNABIONIDS WITH ETHER TYPE SIDE CHAINS*

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The elimination or alleviation of acute pain, arising from cancer, operations, or the spasm of smooth muscle has ever been a challenge to medicinal chemistry. Unfortunately all the s.c. major analgesics, i.e. those which are suitable to eliminate such pains, are at the same time narcotics having more or less severe side effects, the most vicious among them being habituation. Up to the present day attempts to dissociate the analgesic and narcotic properties of a major analgesic have failed.

Marihuana, or hashish, is a relatively harmless drug usually taken in by smoking. One of the features distinguishing it from the so-called hard drugs is that its use does not result in the development of tolerance or physical dependence. Marihuana is a complex mixture of benzopyrane type compounds, the main components of which are Δ^8-and Δ^9-tetrahydrocannabinol (Fig. 1). Both compounds proved to be mild analgesics in certain animal tests, while in others they were ineffective. Tetrahydrocannabinols show some structural resemblance to morphine inasmuch as they contain two saturated or partially saturated six-membered rings which are condensed to a benzene ring. It is remarkable that they do not contain nitrogen. In the organism cannabinoids undergo oxidative metabolism and it has been supposed that some of the metabolites might be active.

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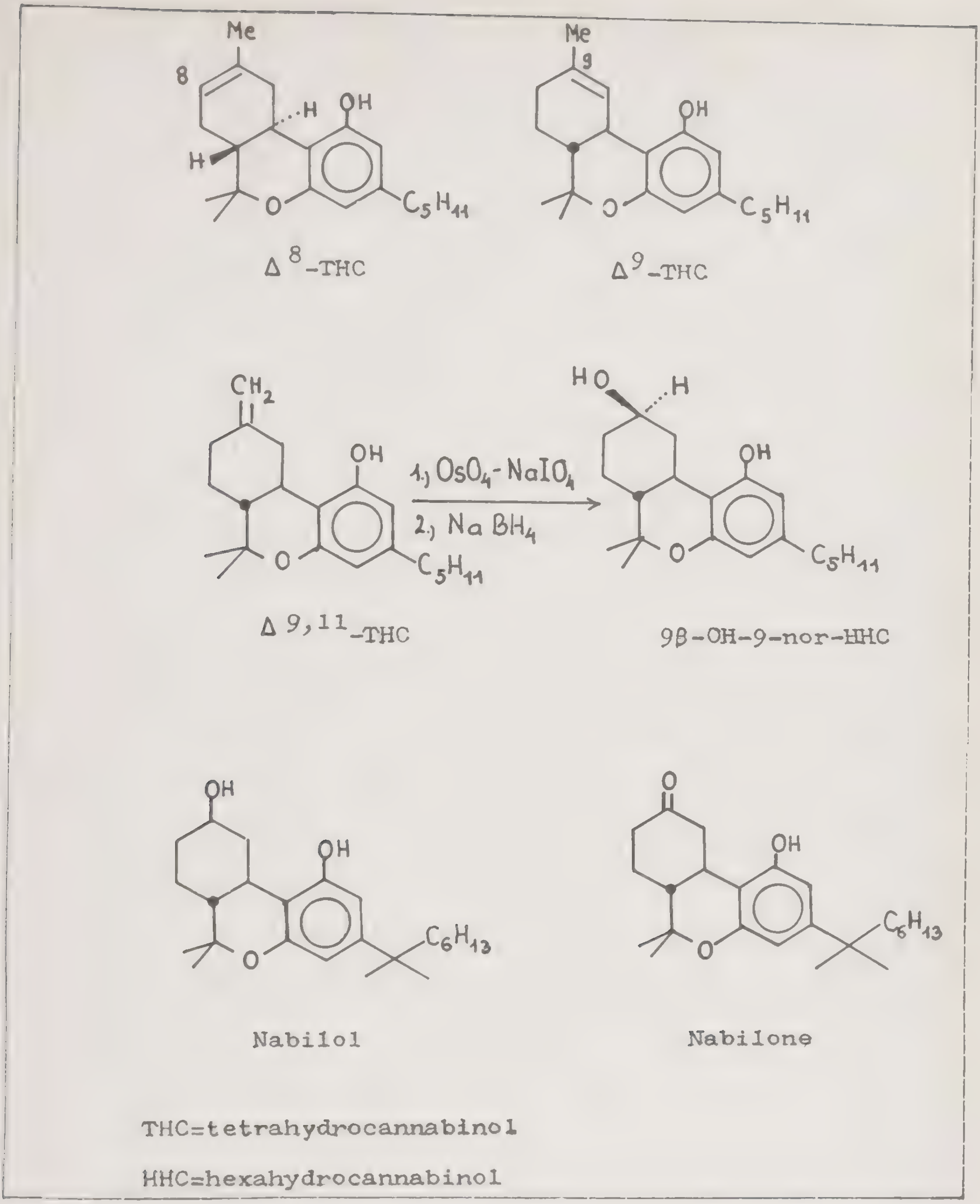
*For references and details, see D. Korbonits, J. Szejtli, Á. Szőke, S. Antus, Á. Gottsegen, M. Nógrádi, Zs. Fürst and J. Knoll, Eur. J. Pharmacol. (in press).

In 1974 Wilson and May prepared by the transformation of a minor cannabis constituent, i.e. of $\Delta^{9,11}$-tetrahydrocannabinol, a non-natural compound called 9-β-hydroxy-9-nor-hexahydrocannabinol (9-β-OH-NHHC).

Later on, in 1976, the compound was examined by the same authors for analgesic activity. The corresponding racemic mixture prepared by synthesis and the α-hydroxy epimer were also tested. The (-)-9β-hydroxy compound turned out to be a very potent analgesic in several tests, while the racemic mixture was only half as active, and the α-epimer was totally inactive. The activity of the (-)-enantiomer paralleled that of morphine. It was also remarkable that the compound did not reverse the withdrawal symptoms of morphine. This indicated that 9β-hydroxy-NHHC may act by another mechanism than morphine. On the other hand naloxon, a well known morphine antagonist, also antagonized 9β-hydroxy-NHHC, which in turn suggested that the two drugs acted on the same receptor.

Concerning side effects no data are available on 9β-hydroxy-NHHC itself, but studies on Δ^9-THC indicated only slight or no depression of respiration, no development of tolerance and very low acute toxicity.



THC=tetrahydrocannabinol

HHC=hexahydrocannabinol

Fig. 1

All these features make 9-hydroxy-9-nor-hexahydrocannabinoids a very promising group of compounds for the development of a non-narcotic and non-addictive major analgesic.

Indeed by a mysterious coincidence of events 15 days before the submission of the paper by Wilson and May describing the preparation of 9β-hydroxy-NHHC, the Eli Lilly Company filed a patent covering the total synthesis of the same compound and of several analogues. It has to be noted that no data for the analgesic properties of 9β-hydroxy--NHHC were published in the patent, but it was claimed that the 1,1-dimethylheptyl analogue, called Nabilol, was a strong analgesic, equipotent to morphine.

These results became known to us at the end of 1975 and one year later we started our own research project with the purpose of preparing and testing original analogues of 9β-hydroxy-NHHC.

In the course of this work we synthesized six new analogues which are shown in Fig. 2. Our compounds are all characterized by one or two ether oxygens in the side chain, but none of them are phenol ethers because these have been shown earlier to be inactive. The synthesis of each and every compound proved to be very difficult and therefore only two of them were subjected to pharmacological testing.

Several methods are available for the synthesis of cannabinoids and we are going to illustrate those which were used in our studies by one example each.

It is noteworthy that the 3-methyl analogue was unknown. We prepared it in six steps by a method developed by Fahrenholz for the synthesis of Δ^9-THC (Fig. 3). The product was extremely insoluble and thus unsuitable for pharmacological testing.

When our work on the methyl compound was already in

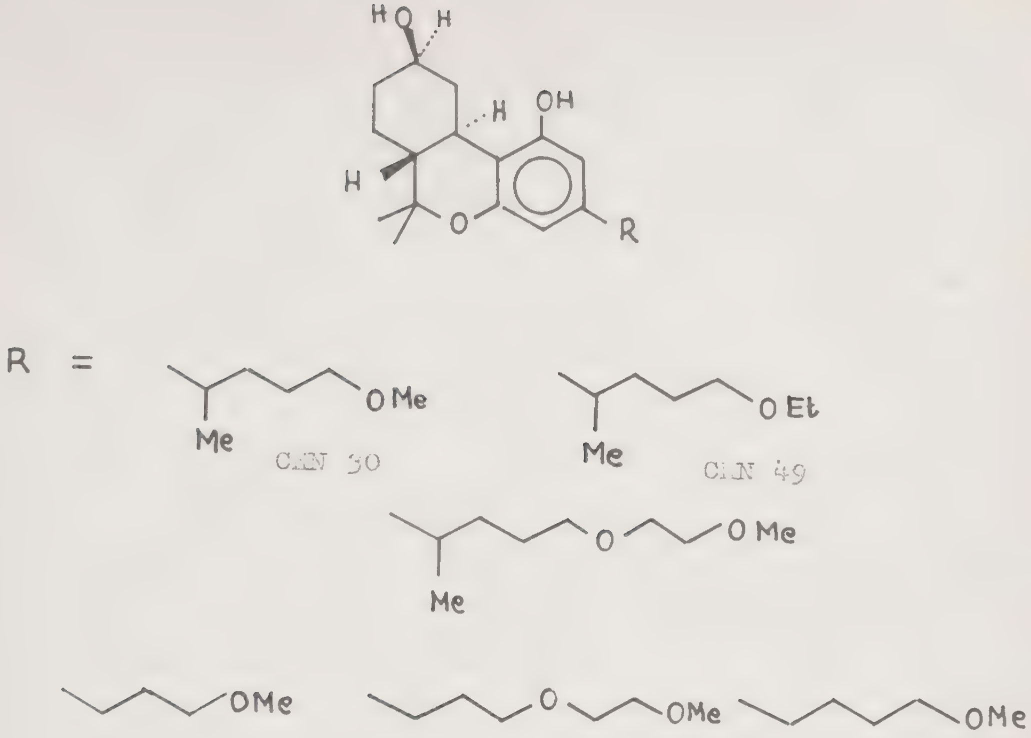
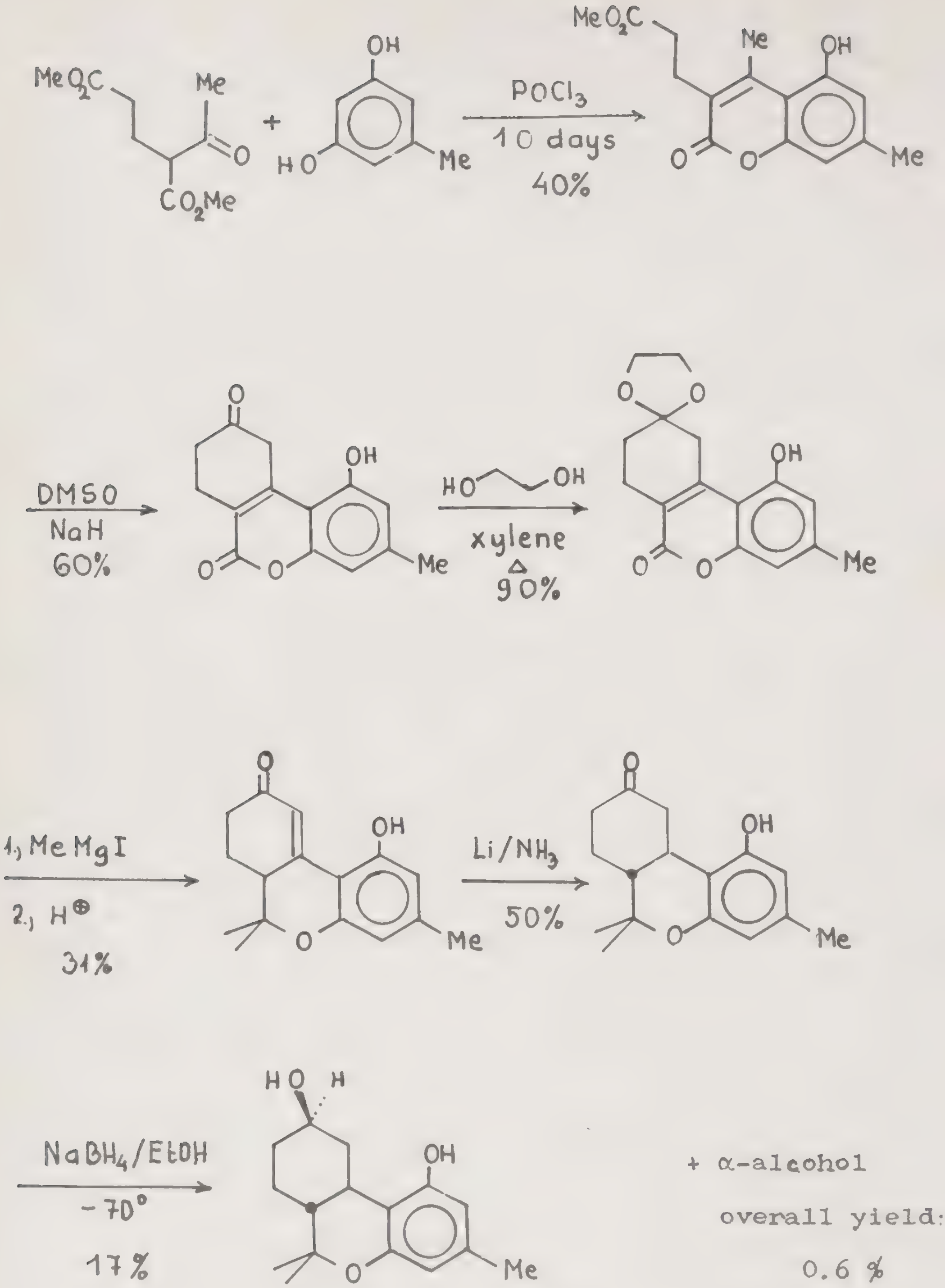


Fig. 2

progress, Archer and his coworkers at the Eli Lilly company published a novel method for the synthesis of the cannabinoid skeleton. We illustrate this approach by the synthesis of two ether analogues of 9β-hydroxy-NHHC (Fig. 4). Comparing the overall yields, the Archer method does not seem better than the Fahrenholz scheme.

We also prepared a series of 5-alkylresorcinols having an ether linkage in the side chain but which did not contain a branching adjacent to the benzene ring (Fig. 5). Condensation of the diphenols with the diene component was successful, but the product could not be transformed to the required trans-ketone, neither by the usual procedure, nor by several other methods. This failure must be connected in some way with the structure of the side chain.



+ α-alcohol

overall yield: 0.6 %

mp. 233-35° C

Fig. 3

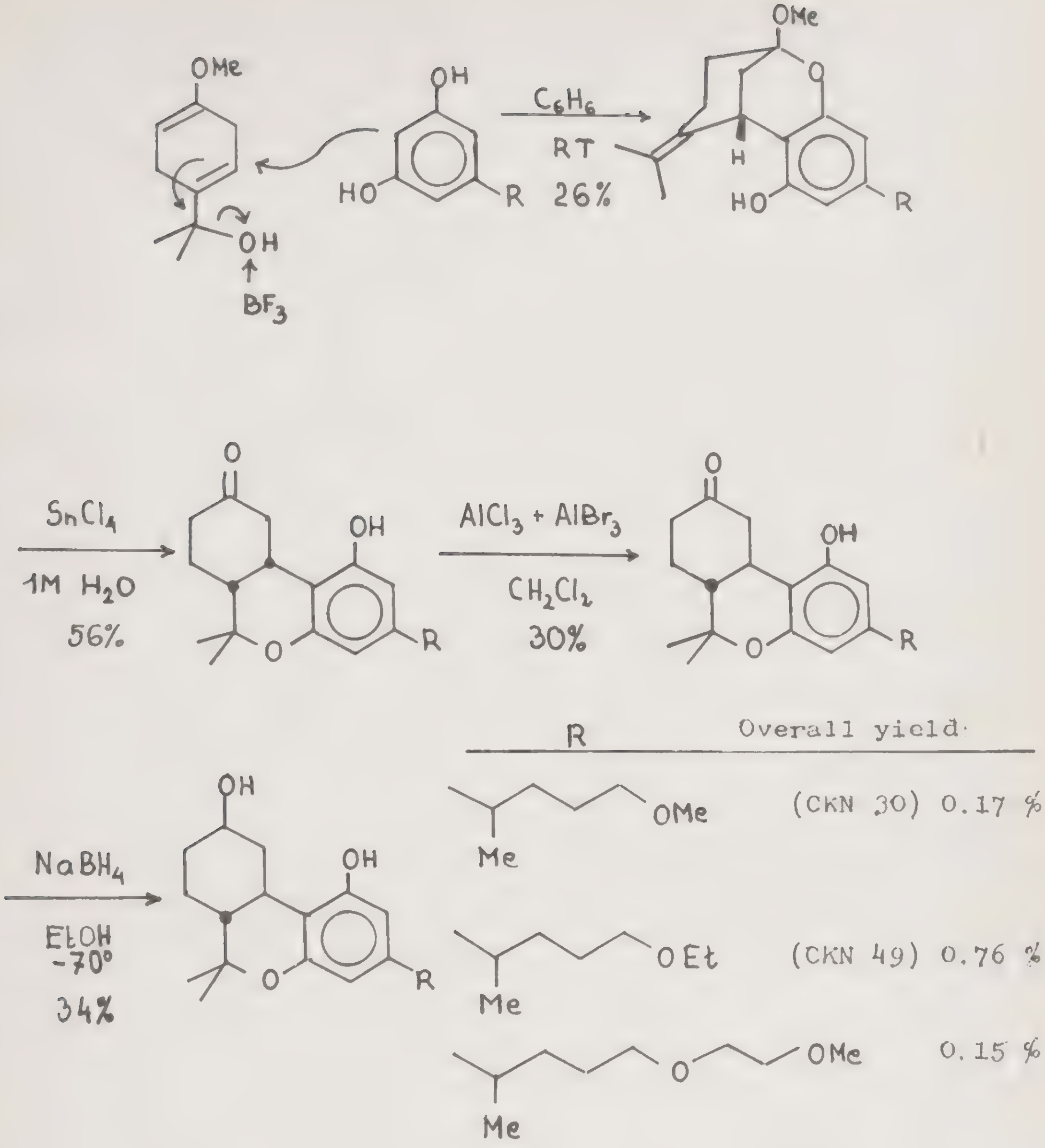


Fig. 4

At this point we became aware of a new patent of the Eli Lilly company, which utilized, as the alicyclic partner, the hydrolysis product of the above mentioned cyclohexadiene. The reaction was catalyzed by tin(IV) chloride. Application

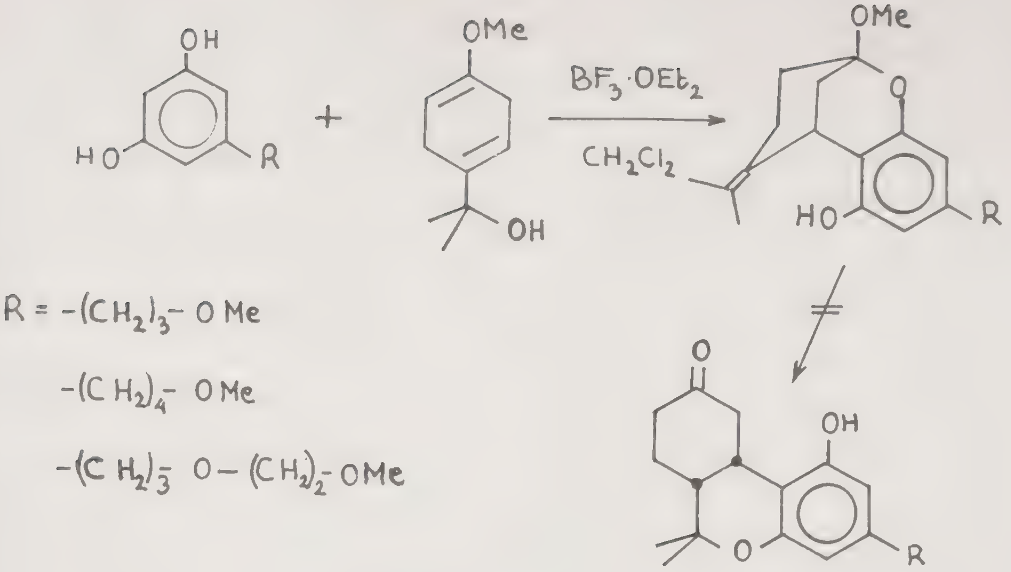

OH
HO
R
OMe
OH
BF3·OEt2
CH2Cl2
OMe
O
HO
R
O
OH
O
R
R = -(CH2)3- OMe
-(CH2)4- OMe
-(CH2)3- O-(CH2)2- OMe

Fig. 5

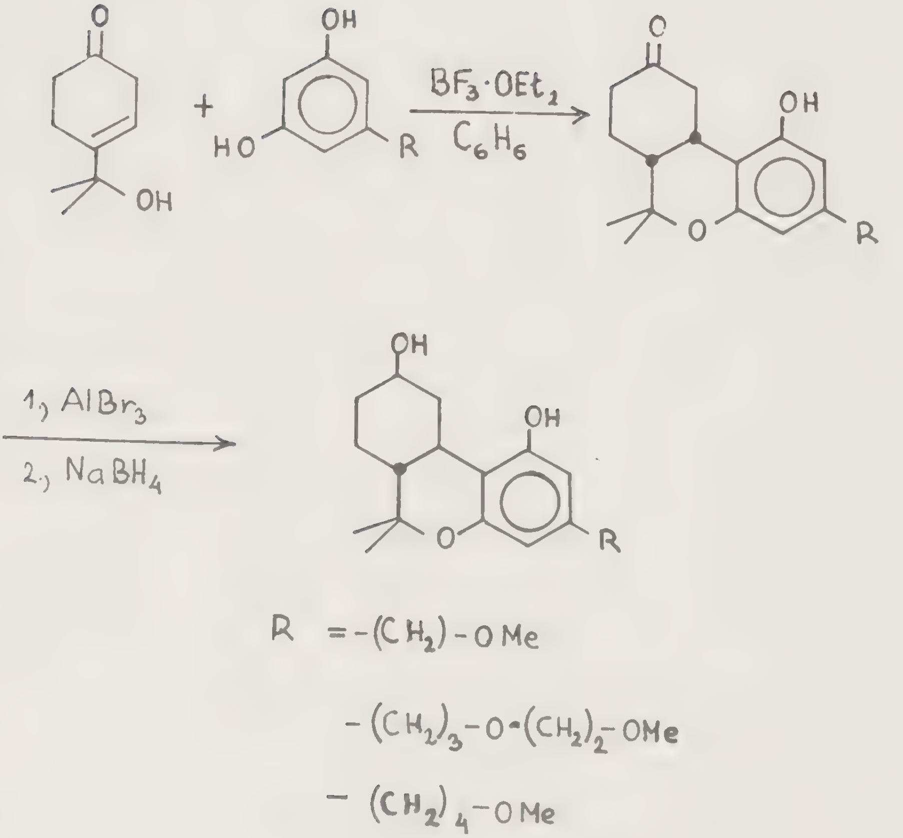

O
OH
OH
HO
R
BF3·OEt2
C6H6
O
OH
O
R
1.) AlBr3
2.) NaBH4
OH
OH
O
R
R = -(CH2)-OMe
-(CH2)3-O-(CH2)2-OMe
-(CH2)4-OMe

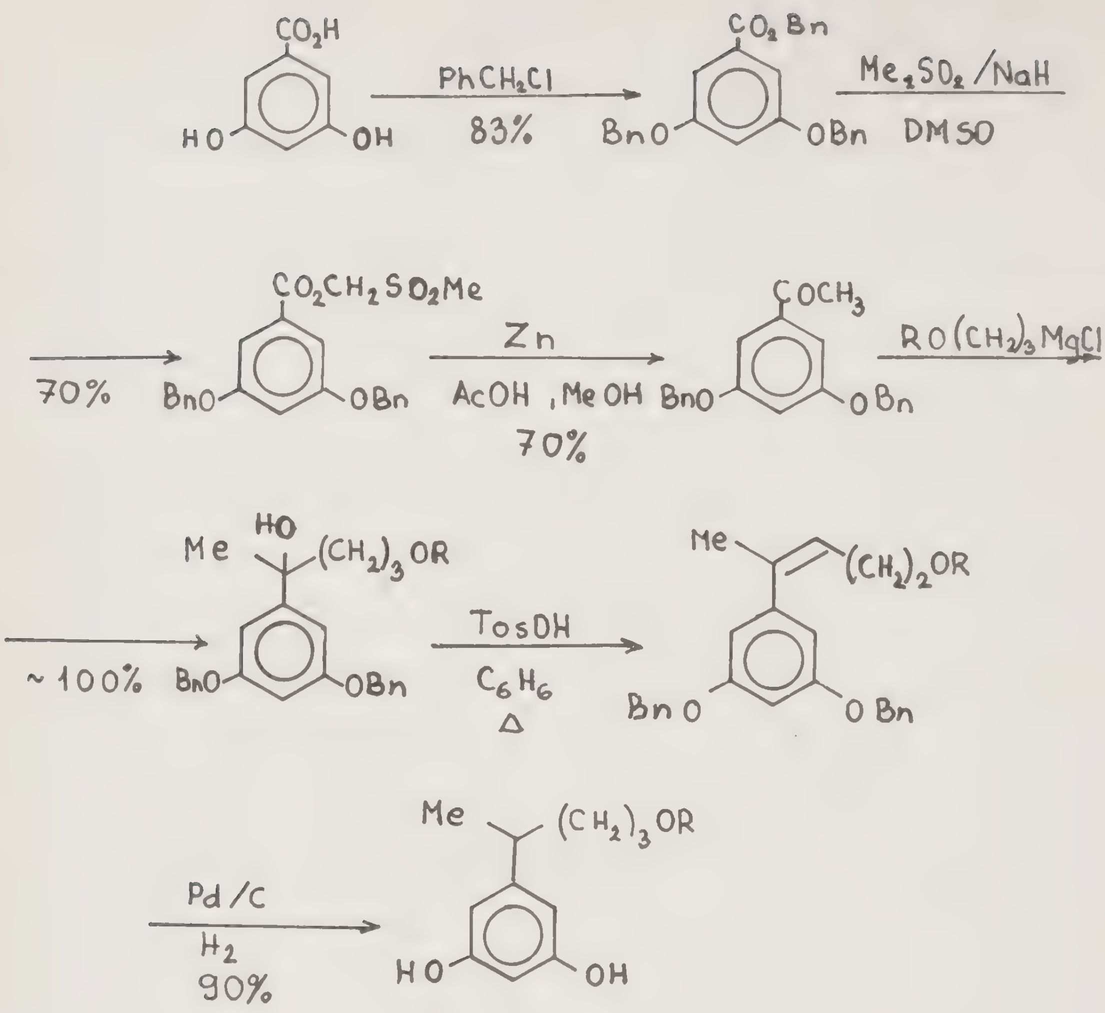
Fig. 6

of the new approach to our resorcinols was, however, disappointing: none of the desired ketones was formed. Interestingly, the use of boron trifluoride was more successful and the cis-ketones were isolated in low yields (Fig. 6). Conversion of the latter to the end products was carried out in the usual way. So far we have not said anything about the resorcinol components. They were, of course, not available commercially and much effort had to be devoted to their synthesis. The preparation of 5-alkyl-resorcinols is not easy in general. Thus e.g. at least five different approaches have been published in the literature for the synthesis of 5-pentylresorcinol.

Our starting material was always 3,5-dihydroxybenzoic acid. The synthesis of the resorcinols with a branched side chain is shown in Fig. 7.

We intended to base the preparation of the resorcinols with unbranched side chain on the Wittig reaction, but this failed completely when the alkyl group contained an ether linkage. After several unsuccessful attempts using other strategies, the final versions are shown in Figures 8 and 9.

Now let us turn to the pharmacological examination of the new compounds. Because of the prohibitive cost of the products only those two were submitted for testing from which it was possible to prepare a few grams by a tolerable investment of work. These were the 5-methoxy- and 5-ethoxy-2-pentyl compounds coded as CKN 30 and 49. As standards 9-β-hydroxy-NHHC and Nabilol were also synthesized. Due to the extremely low solubility of the whole series of compounds in water, pharmacological testing ran into serious difficulties. In the

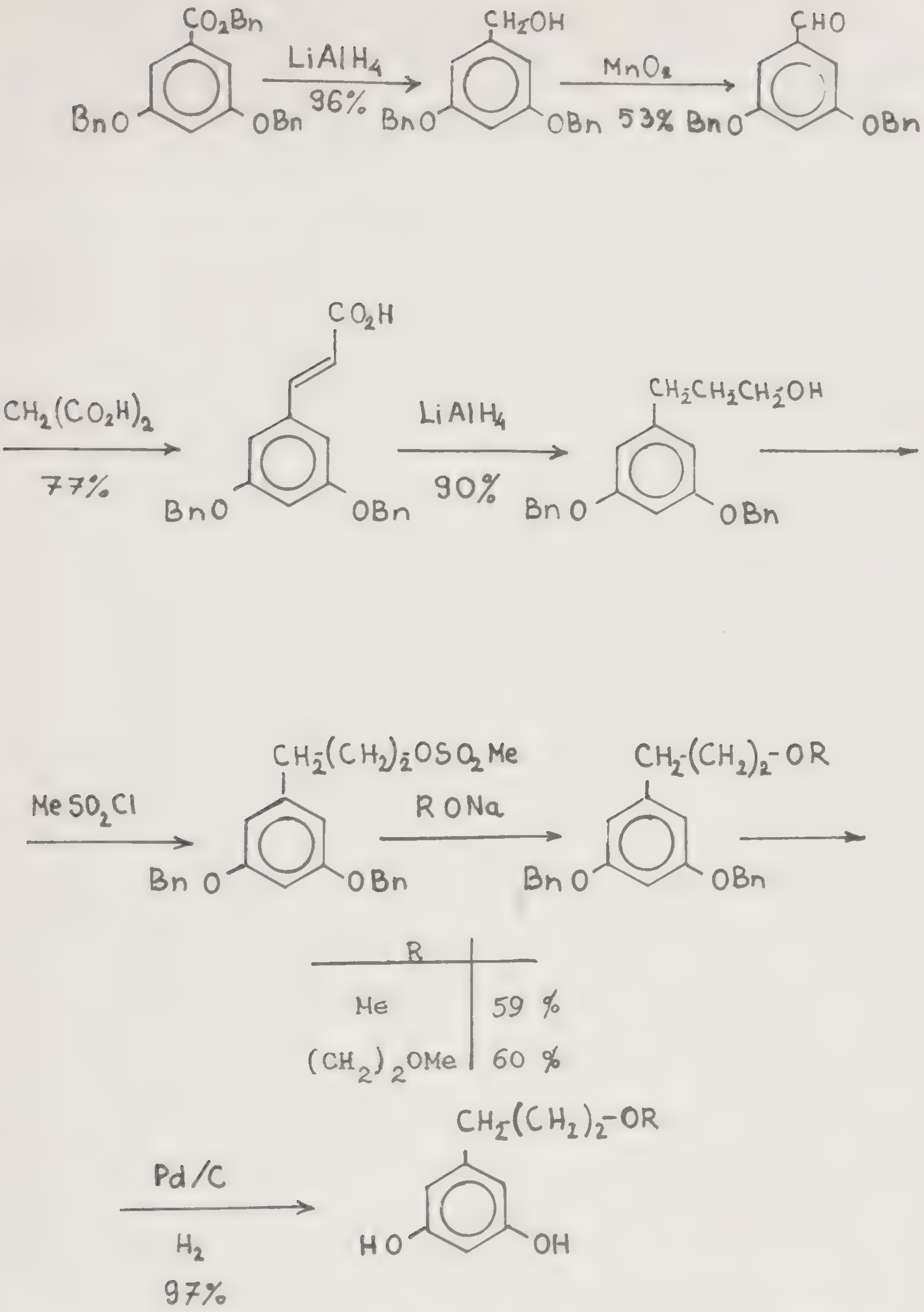


Overall yield: 36 %

$$Cl-(CH_2)_3-Br + NaOR \longrightarrow Cl-(CH_2)_3-OR$$

R	yield
Me	33 %
Et	11 %
$(CH_2)_2OMe$	26 %
with ph. tr. cat.	23 %

Fig. 7



R	
Me	59 %
$(CH_2)_2OMe$	60 %

Overall yield from 3,5-dihydroxybenzoic acid: 16 %

Fig. 8

literature various solubilizing and emulsifying agents were recommended which were claimed to enable the administration of cannabinoids. In our hands, however, all these gave erratic results.

Fortunately, during the same period intensive research was being pursued in the laboratories of Dr. Szejtli at Chinoin on the application of 2,6-di-O-methyl-β-cyclodextrin for the solubilization of hydrophobic drugs (Fig. 10). It is well known that β-cyclodextrin has a hydrophobic cavity which readily includes hydrophobic molecules provided that their size is matching that of the cavity. Complexes of the unsubstituted β-cyclodextrin are, however, only moderately or poorly soluble in water. The reason for this are the strong intermolecular hydrogen bonds established between the free hydroxy groups. If we block two of the three free hydroxyl groups by methylation, solubility in water increases sharply. Such a product is 2,6-di-O-methyl-β-cyclodextrin (DIMEB). DIMEB is capable of complexing cannabinoids and the resulting stable aqueous solutions can be successfully administered to experimental animals. In fact, not only our own new cannabinoids could be complexed by DIMEB but 9β-hydroxy-NHHC, Nabilol and morphine base too.

Results obtained in the hot-plate test in rats were surprizing and are shown in Table 1. May I call your attention to the following points:

1) All the cannabinoids tested were more active than morphine.
2) Both the 5-ethoxy-2-pentyl compound and Nabilol were highly active, i.e. 36-113 times more active than uncomplexed morphine hydrochloride.

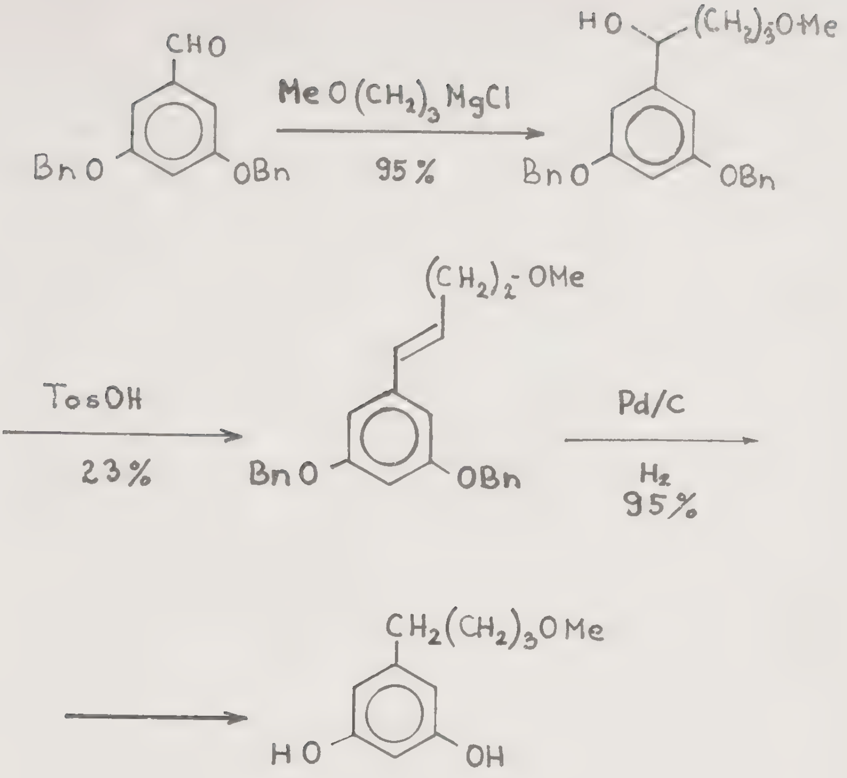
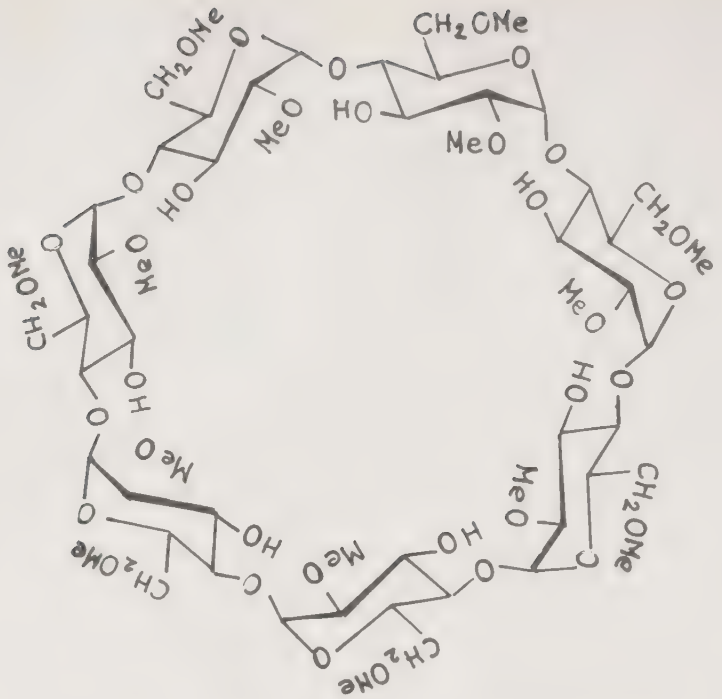


Fig. 9

Table 1. Analgesic activity of some cannabinoids and morphine in the hot plate test in rats

Compound	ED_{50}	
	i.v.	s.c.
9β-OH-NIHHC·DIMEB	0.6	0.9
CKN-30·DIMEB	0.25	0.6
CKN-49·DIMEB	0.022	0.074
Nabilol·DIMEB	0.015	0.025
morphine·DIMEB	0.9	5.0
morphine·HCl	1.7	2.7



Overall yield from 3,5-dihydroxybenzoic acid: 9 %

Fig. 10

3) On intravenous administration complexed morphine base was more active than uncomplexed morphine hydrochloride.

Acute toxicity of our compounds was moderate, i.e. LD_{50} was 22 and 30 mg/kg, resp. Nabilol with an LD_{50} of 8.2 mg/kg was more toxic.

So far so good, but unfortunately it is unlikely that any of the cannabinoids prepared up to the present would find their way as analgesics into clinical practice. Our compounds are too expensive and Nabilol, the compound patented by Eli Lilly seems to have some weakness too. It must be namely not without reason that, apart from the first patent, no reports became public about its use as an analgesic.

This is all the more surprising because its ketone precursor, Nabilone, is in an advanced stage of development as an anti-emetic for use in the prevention of radiation sickness.

Thus the search for a non-addictive major analgesic must be continued.

Flavonoids and Bioflavonoids, 1985
(Eds. L. Farkas, M. Gábor, F. Kállay)

SYNTHESIS OF AMERICANIN-D

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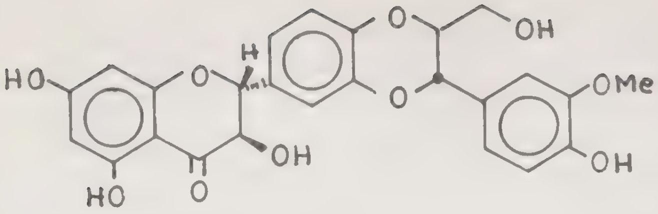
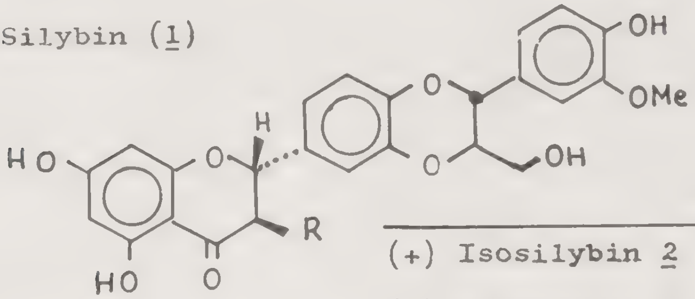
The chemistry of the natural polyphenols occurring in the fruit of *Silybum marianum* Gaertn. /1/ has been the subject of numerous investigations since the discovery of the antihepatotoxic activity /2/ of one of the constituents, the flavanolignan silybin (**1**) /3-6/. Besides silybin the crude extract contains three products, *viz.* silydianin (**4**) /7/, silychristin (**6**) /8/ and isosilybin (**2**) /9/, apparently derived from different modes of coupling of dihydroquercetin and coniferyl alcohol /10/. A few years ago some 3-deoxy derivatives, i.e. silandrin (**3**), silymonin (**5**) /11/, silyhermin (**7**), neosilyhermin-A (**8**) and neosilyhermin-B (**9**) /12/ were isolated in Hungary from the seeds of the white-blooming variety of *Silybum marianum*.

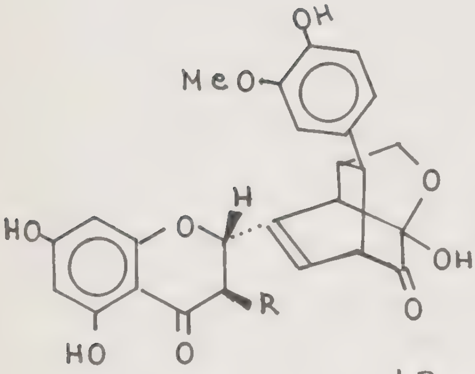
The constitution of silandrin (**3**) could be determined /11/ by correlation with americanin-A (**10**), isolated from *Phytolacca americana* /13/. As a minor constituent of this plant a new lignan, americanin-D (**11**) was isolated by one of us /14/; its structure has been established by comparison of



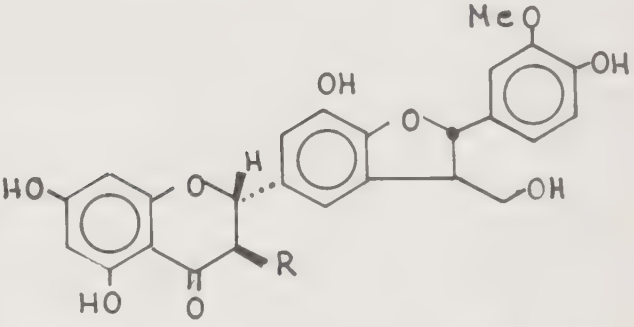
(+) Silybin (1)



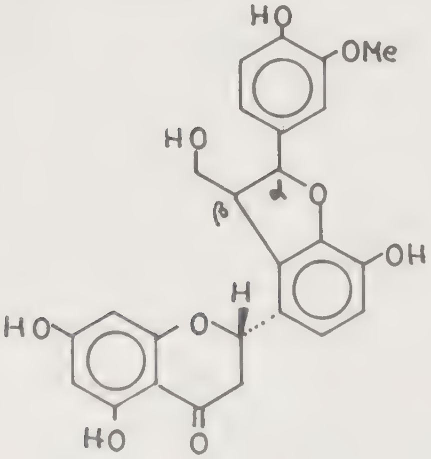
	R
(+) Isosilybin 2	OH
(−) Silandrin 3	H



	R
(+) Silydianin 4	OH
(+) Silymonin 5	H

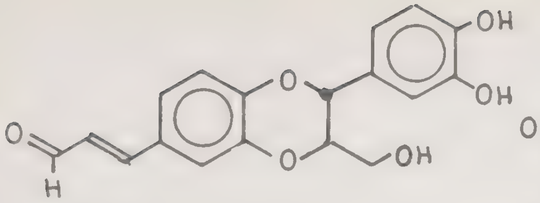


	R
(+) Silychristin 6	OH
(+) Silyhermin 7	H

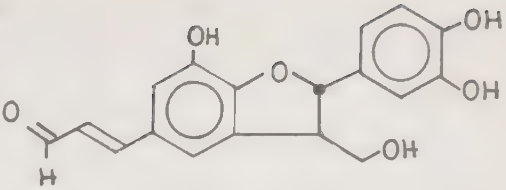


(+) Neosilyhermin-A (8) αR,βS or αS,βR

(+) Neosilyhermin-B (9) αS,βR or αR,βS



Americanin-A (10)

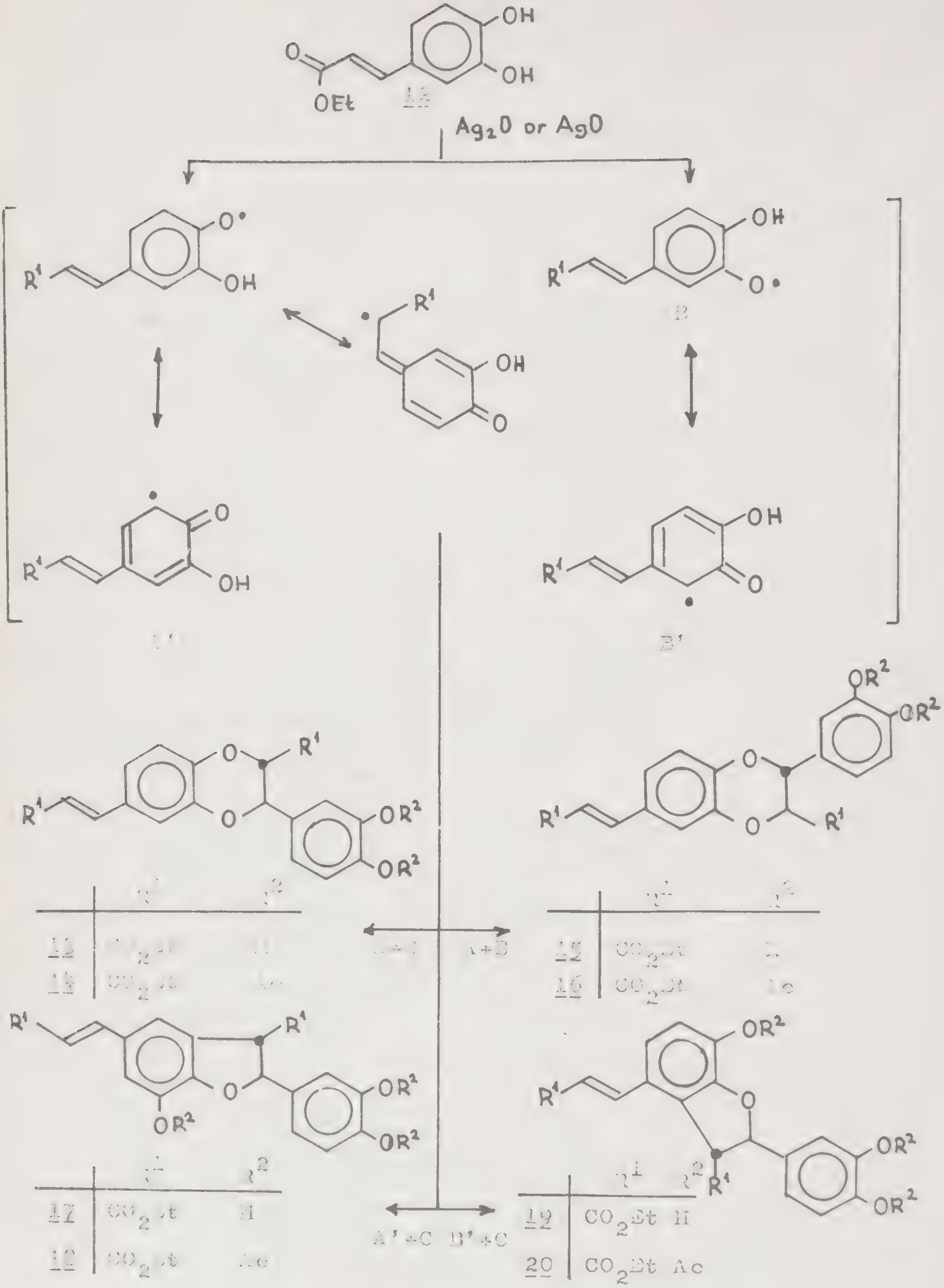


Americanin-D (11)

its ^{13}C- and ^{1}H-NMR data with those of silychristin (6).

The synthesis of americanin-D is of double interest, since it could serve as a building block for the preparation of silychristin-type compounds according to the method of Antus et al. /15/; also, the synthesis of americanin-D can provide an opportunity to examine the scope and limitation of the oxidative phenol coupling method of Merlini et al. /16/, as it remained unexplained why the reaction of substituted catechols with coniferyl alcohol gave always only the 2,3-trans-benzodioxans. A free-radical coupling mechanism was proposed for this reaction. Since in Merlini's experiments coniferyl alcohol was a constant partner and only the catechols were different (having electron-donating or -withdrawing substituents, thereby influencing regioselectivity), it was of interest to use, instead of coniferyl alcohol, some cinnamoyl esters, e.g. the ethyl ester of caffeic acid (12).

When reacting 12 under the conditions used by Merlini /16/, we obtained a complex mixture. HPLC analysis revealed incomplete conversion (32 % of 12) and the presence of 34 new compounds, from which two types (13/15 and 17/19) repre-



	R¹	R²
13	CO_2Et	H
14	CO_2Et	Ac

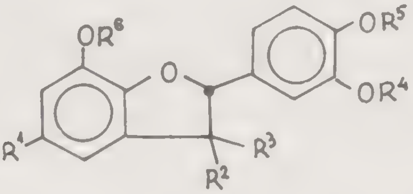
	R¹	R²
15	CO_2Et	H
16	CO_2Et	Ac

	R¹	R²
17	CO_2Et	H
18	CO_2Et	Ac

	R¹	R²
19	CO_2Et	H
20	CO_2Et	Ac

sented 44 %, while the percentage of the remaining 32 products was ~ 1 % each.

The reaction mixture was acetylated. Preparative chromatography of the crude acetate gave two main components in a ratio 4:1. According to spectroscopic data, the major product proved to be a benzofuran derivative (18 or 20). In order to distinguish between these regioisomers, we have transformed our acetate in five steps (21, 22, 23, 24, 25) into the dihydrodehydrodiisoeugenol methyl ether (25) of established structure /17/.



	R^1	R^2	R^3	R^4	R^5	R^6
21	$(CH_2)_2CO_2Et$	H	CO_2Et	Ac	Ac	Ac
22	$(CH_2)_2CH_2OH$	H	CH_2OH	H	H	H
23	$(CH_2)_2CH_2OH$	H	CH_2OH	Me	Me	Me
24	$(CH_2)_2CH_2OTs$	H	CH_2OTs	Me	Me	Me
25	$(CH_2)_2CH_3$	H	CH_3	Me	Me	Me
26	(E)-$CH{=}CH{-}CO_2Et$	H	CO_2Et	$MeOCH_2$	$MeOCH_2$	$MeOCH_2$
27	(E)-$CH{=}CH{-}CO_2Et$	H	CO_2Et	$MeOCH_2$	H	$MeOCH_2$
28	(E)-$CH{=}CH{-}CO_2Et$	H	CO_2Et	H	$MeOCH_2$	$MeOCH_2$
29	(E)-$CH{=}CH{-}CO_2Et$	H	CH_2Et	$MeOCH_2$	$MeOCH_2$	$MeOCH_2$
30	(E)-$CH{=}CH{-}CH_2OH$	H	CH_2OH	$MeOCH_2$	$MeOCH_2$	$MeOCH_2$
31	(E)-$CH{=}CH{-}CHO$	H	CH_2OH	$MeOCH_2$	$MeOCH_2$	$MeOCH_2$

The literature recommends several methods (LAH, DIBAH) /18, 19/ for the reduction of both ester functions to hydroxyls and α,β-unsaturated esters to α,β-unsaturated aldehydes. Unfortunately, treatment of our acetate with LAH or DIBAH at low temperature gave a polymer. Therefore the reduction of 18 to americanin-D (11) had to be carried out in several steps: (i) deacetylation, (ii) protecting of the phenolic hydroxyls, (iii) reduction of the ester groups to alcohols, (iv) oxidation of the allylic alcohol to aldehyde and, finally, removal of the protecting groups.

Since americanin-D (11) contains a trans-cinnamaldehyde side chain as well as a catechol unit, both being sensitive to oxidation, it was evident that the key to the realization of the above-mentioned scheme was to find a suitable protecting group.

Our previous experiences /20, 21/ suggested that the methoxymethyl group would fulfil the requirements. Accordingly, 18 was transformed to the phenol 17 by mild acid hydrolysis, methoxymethylated to 26, reduced with LAH at -15 °C to the corresponding alcohol (30), in which the allylic alcohol group was oxidized to give 31. This compound, when treated under extremely mild acidic conditions (3×10^{-2} mol/l hydrochloric acid in methanol) furnished, after deprotection, americanin-D (11). All spectra of 11 proved to be identical with those of the natural product.

The success of the synthesis confirmed the feasibility of the previously postulated sequence, and confirmed the usefulness of the methoxymethyl protecting group. What could not be anticipated was the difficulty of the methoxymethylation step. We were surprised to find that within a very short time the C-hydroxymethyl compound 29 was formed in 71 % yield. Compound 29 is evidently a secondary product, derived from the required 26. Decreasing the reaction time to 10 minutes, about 50 % of 26 was detected, while the quantity of 29 was reduced to 8 %. The partially alkylated derivatives 27 and 28 amounted to a total of 26 %.

Compounds 27 and 28 are valuable intermediates for the synthesis of the silychristin-type flavanolignans 6, 7, 8 and 9.

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SYNTHESIS OF SOME NOVEL FLAVONOID DERIVATIVES

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4-Aminoflavan (1a) was first prepared by our research team in 1959 /1-3/; later 4-amino-1-thiaflavan (1b) /4,5/ was also synthesized, and the 2,4-*cis*-configuration /6,7/ of both compounds, as well as their absolute configurations /8/ were elucidated.

1	a	b
X	O	S

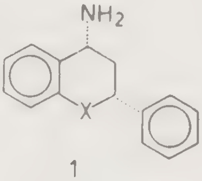

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Fig. 1

Experiments were also made /9/ to synthesize the 2,4-*trans* isomer of 4-aminoflavan. Based on literature data /10/, in the present work the Leuckart-Wallach reaction of flavanone (2a) and 1-thiaflavanone (2b) was studied.

Noyce and Bachelor /10/ reported that in the product of the Leuckart-Wallach reaction of cyclic ketones carrying an alkyl group adjacent to the carbonyl function, the *trans*-amine predominated over the *cis* isomer to a larger extent than in the product mixture obtained by means of the catalytic hydrogenation of oximes; further, it has been found that the bulkier the alkyl group, the higher the proportion of the *trans*-amine. These authors also recognized an analogy between the transition state of the third step of the Leuckart-Wallach reaction and that of the Meerwein-Ponndorf-Verley reduction. This finding is of interest, as this latter type of reduction of flavanone (2a) was found, by us, to lead to 2,4-trans-4-hydroxyflavan.

Flavanone (2a) was melted together with five mole-equivalents of ammonium formate and the volume of water, produced in the reaction, was found to be several times higher than that expected on the basis of the Leuckart-Wallach reaction. This finding suggested that additional condensation and oxidation reactions had occurred.

From the alcohol-soluble part of the solid product, precipitated from the reaction mixture, 4-formamidoflavan - produced in the Leuckart-Wallach reaction - was isolated and converted by hydrolysis with hydrochloric acid into 4-aminoflavan hydrochloride (1a.HCl). This compound, obtained in 6.3% yield, was shown to be identical with 2,4-cis-aminoflavan hydrochloride (1a.HCl) prepared by the catalytic hydrogenation of flavanone oxime.

Further examinations were directed to elucidate the structure of the new, slightly alcohol-soluble compound, isolated in a yield of 17.6%. The IR spectrum showed the presence of chelated hydroxyl group and two mono- and two di-(ortho)-substituted aromatic rings in the molecule, as well as the absence of carbonyl function. Of the CH frequencies the aromatic bands were predominant.

On the basis of ^{1}H-NMR spectral and elemental analytical data, 19 of the 21 protons are attached to aromatic rings. The proton signal appearing at δ 14.6 ppm corresponds to a hydrogen-bonded, chelated hydroxyl group. A molecular weight of 427 and molecular formula $C_{30}H_{21}NO_2$ were established by mass spectrometry and elemental analyses. Acetylation of the substance with acetic anhydride in pyridine gave a single O-monoacetyl derivative. On this basis, for the new product structure 9 seemed probable (Fig. 3).

The above results and other chemical considerations suggest the following mechanism for the formation of 2,4-cis-4-aminoflavan hydrochloride (1a.HCl): (Fig. 2).

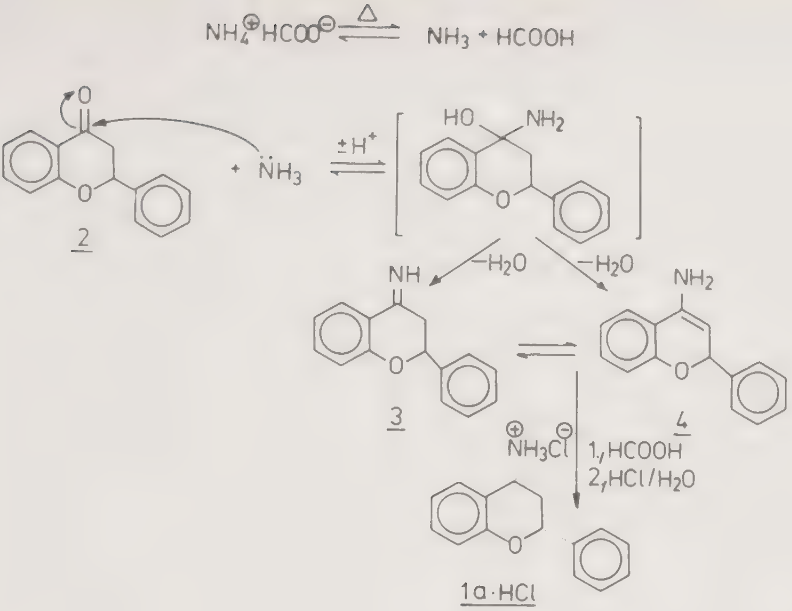

Fig. 2

The formation of the new compound 9 can be explained by a Michael addition reaction between the enamine 4 and 2'-hydroxychalcone (5), both arising under the given reaction conditions

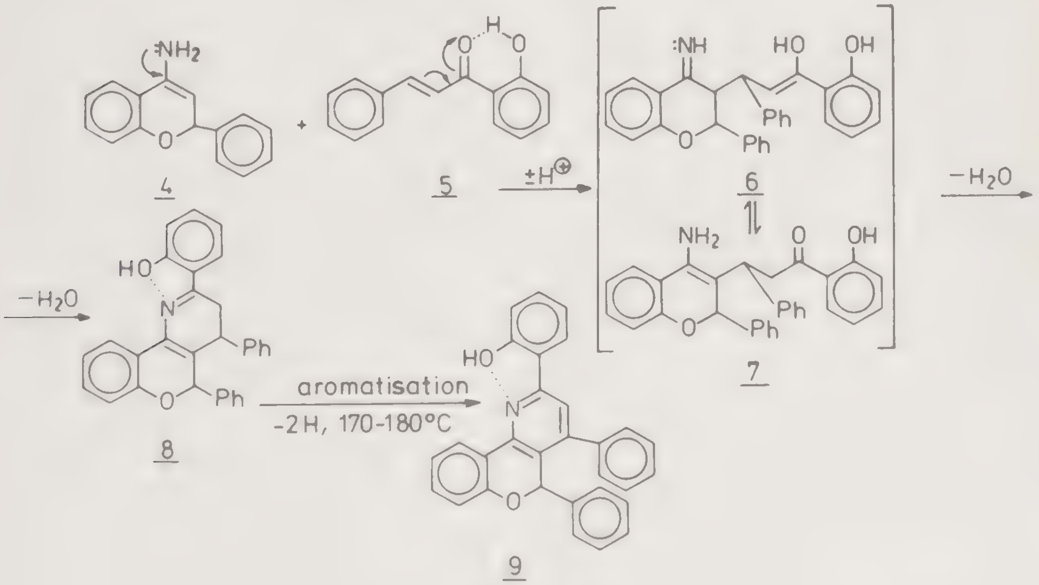

Fig. 3

(the latter by ring-opening of flavanone 2), and subsequent condensation of the intermediary 6-7 into 8, followed by aromatization (Fig. 3).

To afford chemical proof for this mechanism of the formation of the benzopyrano-pyridine derivative 9, a multi-step synthesis leading to 2,4-diphenyl-5,6,7,8-tetrahydroquinoline (17) was devised, where, instead of flavanone (2) and 2'-hydroxychalcone (5), cyclohexanone (10) and chalcone (13), respectively, were applied. The first step, the reaction of cyclohexanone (10) and morpholine (11), in the presence of p-toluenesulphonic acid, gave morpholinocyclohexene 12:

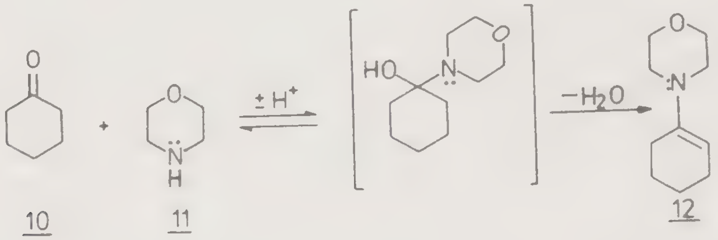

Fig. 4

Michael addition of this enamine (12) (purified by vacuum distillation) to chalcone (13) in chloroform resulted in the iminium compound 14, from which the 1,5-dioxo analogue 15 was

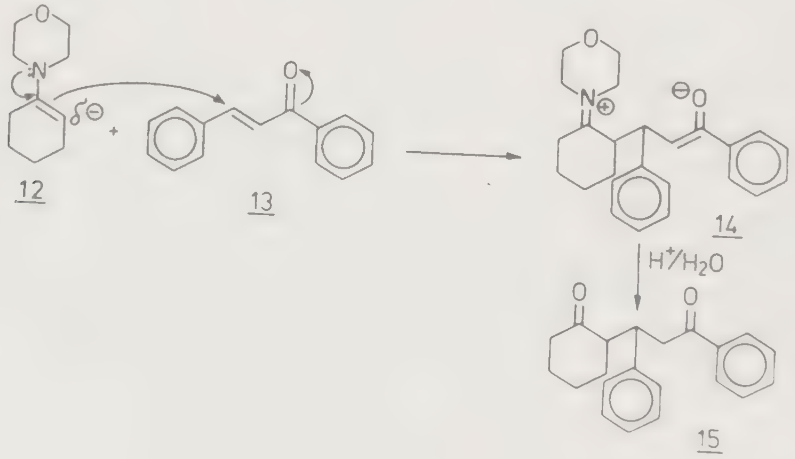

Fig. 5

prepared by treatment with hot hydrochloric acid (Fig. 5).

The reaction of the diketone 15 with an excess of ammonium acetate yielded the enamine 16 which, on ring-closure and dehydrogenation, transformed into compound 17 containing the pyridine ring structure:

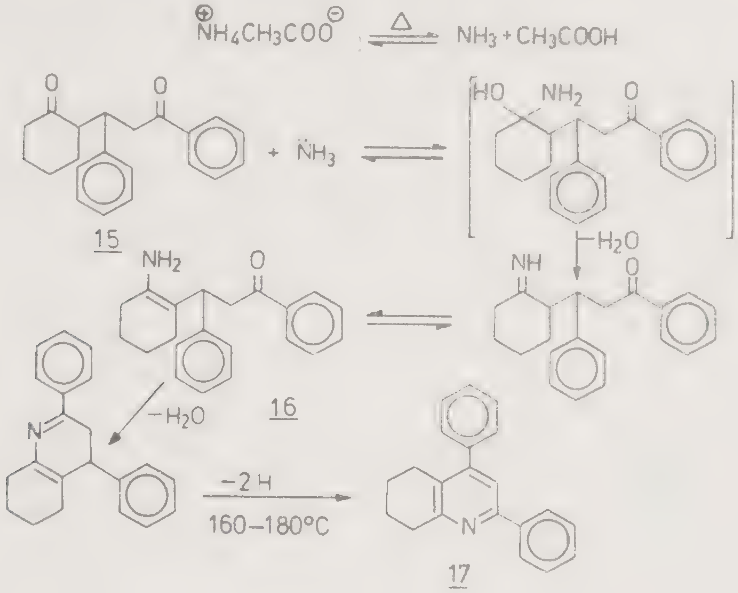
$\overset{\oplus}{N}H_4CH_3CO\overset{\ominus}{O}$ $\overset{\Delta}{\rightleftharpoons}$ $NH_3 + CH_3COOH$
15
16
17
$-H_2O$
$-H_2O$
$-2H$
160–180°C

Fig. 6

This synthetic route clearly shows that 1,5-diketones are readily transformed into compounds with pyridine-type ring system when treated with ammonium salts (acetate, formate).

However, flavanone could not be transformed into an isolable morpholino- or piperidino-enamine derivative corresponding to 12, and being suitable for conversion to the 1,5-diketone analogues. As the benzopyrano-pyridine ring structure can be generated from both the enamine and the 1,5-diketone, it is difficult to decide whether the formation of the 1,5-diketone - the product of the Michael-addition - precedes the production of the emanine or not. In the case of flavanone, however, primary formation of the enamine seems more probable.

In subsequent experiments the benzopyrano-pyridine analogues were synthesized by using ammonium acetate. Flavanone (2a) and an excess of ammonium acetate were melted together, and 2'-hydroxychalcone (5) was added to the melt. The product, 2-(2'-hydroxyphenyl)-4,5-diphenyl-5H[1]-benzopyrano-[4,3-b]-pyridine (9) could be isolated from the mixture:

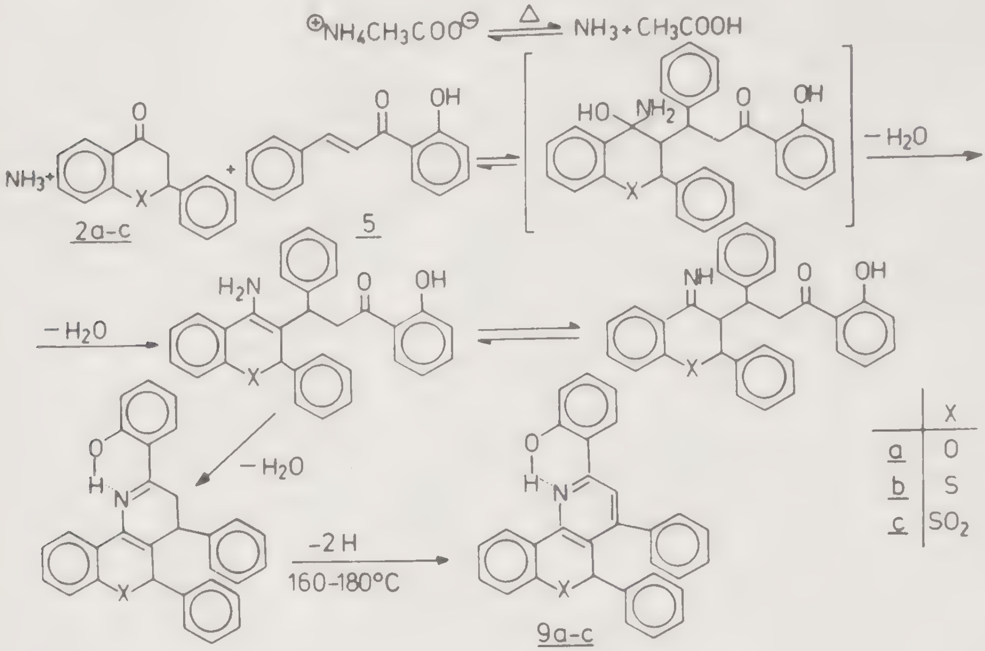

Fig. 7

Extension of this method to thiaflavanone (2b) and thiaflavanone-1,1-dioxide (2c) allowed the preparation of 2-(2'-hydroxyphenyl)-4,5-diphenyl-5H-[1]-benzothiapyrano-[4,3-b]-pyridine (9b) and its 1,1-dioxide analogue (9c). Acetylation of the phenolic hydroxyl group of these derivatives gave, again, the corresponding O-monoacetates in quantitative yield.

The above experiments unequivocally prove that 2'-hydroxychalcone takes part, indeed, in the reactions leading to the benzopyrano-pyridine- and benzothiapyrano-pyridine derivatives.

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SYNTHESIS OF BENZOPYRANO[4,3-c]PYRAZOLINES AND RELATED COMPOUNDS

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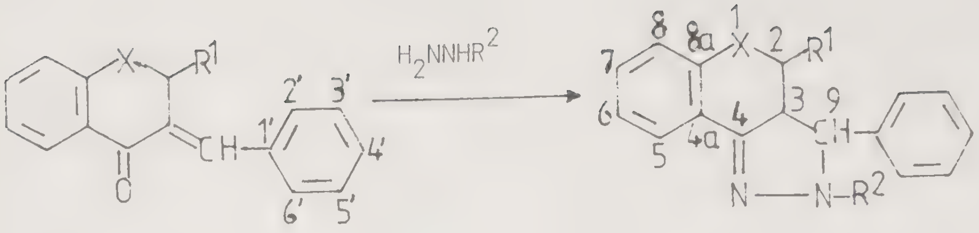
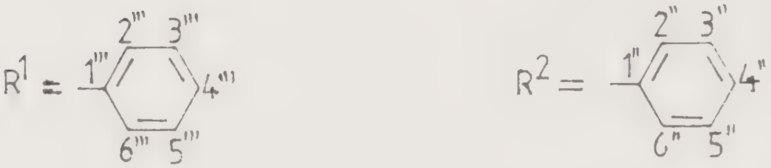
Benzopyranopyrazolines were synthesized by intramolecular cycloaddition of nitrile imides or nitrilimines (1-4). The overall yields of these reactions were, however, quite low (approx. 20%) and the stereochemistry of the benzopyranopyrazolines prepared in this way has not been investigated. These facts prompted us to devise another synthetic route for the preparation of such fused heterocycles and to investigate the stereochemistry of the compounds synthesized. 3-Benzylidenechromanones, -1-thiochromanone and 2-benzylidene-1-tetralone proved to be convenient synthons for these syntheses. The E-isomers of these substances were prepared by the condensation of the corresponding ketone and aldehyde components in the presence of piperidine (5,6).

Synthesis of pyrazolines by the reaction of α,β-unsaturated ketones and hydrazines is well-known (7-10). Pyrazolines possessing fused ring system have been synthesized by the reaction of exocyclic α,β-unsaturated ketones and hydrazines (11, 12). Further utilization of this procedure is reported in the present paper.

The α,β-unsaturated ketones 1 - 4 were refluxed for 6 h in pyridine with methyl- or phenylhydrazine to give the pyrazolines 5 - 12 (Table 1). Concerning the reaction mechanism, we suppose that the first step is a Michael addition to the β-carbon atom followed by a nucleophilic addition and elimination to form pyrazoline ring.



1 - 4   5 - 12



1: X = CH_2, R^1 = H

2: X = S, R^1 = H

3: X = O, R^1 = H

4: X = O, R^1 = C_6H_5

5: X = CH_2, R^1 = H, R^2 = CH_3

6: X = CH_2, R^1 = H, R^2 = C_6H_5

7: X = S, R^1 = H, R^2 = CH_3

8: X = S, R^1 = H, R^2 = C_6H_5

9: X = O, R^1 = H, R^2 = CH_3

10: X = O, R^1 = H, R^2 = C_6H_5

11: X = O, R^1 = C_6H_5 R^2 = CH_3

12: X = O, R^1 = C_6H_5 R^2 = C_6H_5

Table 1

Physical constants and analytical data of compounds prepared

	M.p., ^{o}C	Yield, %	Overall formula	Analysis, %					
				Calculated			Found		
				C	H	N	C	N	H
5	137 - 138	63.7	$C_{18}H_{18}N_2$	82.44	6.87	10.68	82.56	6.89	10.80
6	149 - 150	46.1	$C_{23}H_{20}N_2$	85.18	6.17	8.64	85.78	6.09	8.41
7	115 - 116	45.4	$C_{17}H_{16}N_2S$	72.85	5.71	9.98	72.35	5.65	9.84
8	156 - 157	69.2	$C_{22}H_{18}N_2S$	77.19	5.26	8.18	77.29	5.28	8.15
9	123 - 124	46.3	$C_{17}H_{16}N_2O$	77.27	6.06	10.60	77.45	6.14	10.69
10	143 - 144	53.8	$C_{22}H_{18}N_2O$	80.98	5.52	8.58	79.58	5.35	8.32
11	161 - 162	55.5	$C_{23}H_{20}N_2O$	81.17	5.88	8.23	81.46	6.09	8.28
12	182 - 183	47.2	$C_{28}H_{22}N_2O$	83.58	5.47	6.96	83.44	5.49	7.17

Table 2

^{1}H-NMR data of compounds 5 - 12

	5	6	7	8	9	10	11	12
δ_{H-1}	2.7 - 2.9	2.7 - 3.0	-	-	-	-	-	-
δ_{H-2}	2.7 - 2.9	1.95/ax/	2.81/eq/	2.97/eq/	4.48/eq/	4.58/eq/	5.05	5.05
		2.25/eq/	3.21/ax/	3.29/ax/	4.13/ax/	4.12/ax/		
δ_{H-3}	3.07	3.25	3.55	3.61	3.53	3.56	3.78	3.67
δ_{H-9}	3.63	4.61	3.79	4.69	3.78	4.65	3.84	4.65
δ_{H-5}	7.90	8.06	7.98	8.04	7.78	7.84	7.86	7.92
δ_{CH_3}	2.81	-	2.78	-	2.81	-	2.78	-
$J_{2,2}$			-11.3 Hz	-12.65 Hz	-10.0 Hz	-10.0 Hz	-	-
$J_{3,2}$	12.5 Hz	12.1 Hz	11.0 Hz	12.65 Hz	11.0 Hz	12.2 Hz	12.1 Hz	10.0 Hz
		4.95 Hz	4.2 Hz	4.95 Hz	5.5 Hz	5.6 Hz		
J [illegible]	13.5 Hz	12.1 Hz	13.2 Hz	11.55 Hz	13.0 Hz	11.7 Hz	12.5 Hz	10.0 Hz

Table 3

^{13}C-chemical shift data of compounds 5 - 12

	5	6	7	8	9	10	11	12
C-1	29.2^{x}	29.4^{x}	-	-	-	-	-	-
C-2	26.7^{x}	27.4^{x}	29.4	29.8	69.3	68.8	83.5	83.1
C-3	54.6	56.4	54.7	55.6	52.4	53.6	58.1	59.7
C-4	152.1	150.5	148.1	146.3^{x}	146.9	146.5	146.7	146.9
C-4a	128.3	128.2	126.9	126.4	116.4	116.1	116.5	116.4
C-5	124.0	124.4	125.4	125.9	124.4	124.5	124.4	124.7
C-6	126.6	126.7	125.1	125.1	121.7	121.6	121.8	121.6
C-7	129.0	129.1	128.3	128.0	131.0	130.9	130.9	130.8
C-8	128.8	129.0	126.9	126.7	117.4	117.2	117.5	117.3
C-8a	139.5	142.2	133.9	133.9	155.7	155.6	156.3	156.1
C-9	80.4	73.3	80.8	73.2	77.6	69.8	77.3	68.7
C-1'	138.1	138.1	138.6	141.3	138.6	141.2	138.4	141.3
C-2',6'	128.7	129.3	128.7	128.7	128.9	128.8	128.0	128.8
C-3',5'	127.4	126.1	127.3	125.9	127.4	126.0	127.6	[illegible]
C-4'	127.8	127.7	128.8	128.0	128.3	127.9	127.4	127.1
C-1''	-	146.9	-	146.1^{x}	-	145.2	-	[illegible]
C-2'',6''	-	114.9	-	114.6	-	114.8	-	114.1
C-3'',5''	-	128.7	-	129.4	-	129.3	-	[illegible]
C-4''		120.0	-	120.2	-	120.7	-	[illegible]
C-1'''	-	-	-	-	-	-	136.1	[illegible]
C-2''',6'''	-	-	-	-	-	-	[illegible]	[illegible]
C-3''',5'''	-	-			-	-	127.6	[illegible]
C-4'''		-		-	-	-	129.1	[illegible]
CH_3	42.0	-	41.9	-	42.0	-	41.9	

^{x}Alternative assignment

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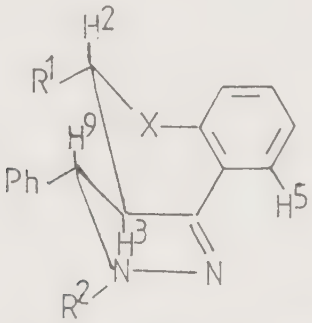
On the basis of the ^{1}H- and ^{13}C-NMR spectra, compounds 5 - 12 were found to consist of one diastereoisomer. The spectral data are summarized in Tables 2 and 3. The aliphatic hydrogens in compounds 7 - 10 showed an ABMX, while those in substances 11 and 12 an ABX spectrum. In the case of compound 5 and 6 the $J_{3,9}$ coupling constant can be determined from the AB part of the relatively more complicated spectra. From the coupling constants measured (10.0 - 13.5 Hz) it can be deduced that H-3 and H-9 are trans oriented and H-3 is axial in the dominant conformer. It can also be established that in compounds 11 and 12 H-2 and H-3 are trans oriented, too, and the phenyl group in position 2 is equatorial. For all these bases the following stereostructure seems to be the most probable.



$J_{3,9}$ = 10.0 - 13.5 Hz
$J_{2ax,3}$ = 10.0 - 12.5 Hz

This structure is corroborated by the fact that H-5 is relatively deshielded (δ 7.78 - 8.06 ppm) as a consequence of the anisotropic effect of the C=N double bond.

For the ^{13}C-NMR assignments known data of tetralone, chromanone, 1-thiochromanone and flavanone (13-16) were utilized.

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SYNTHESIS OF NATURAL PYRANOCHROMONES

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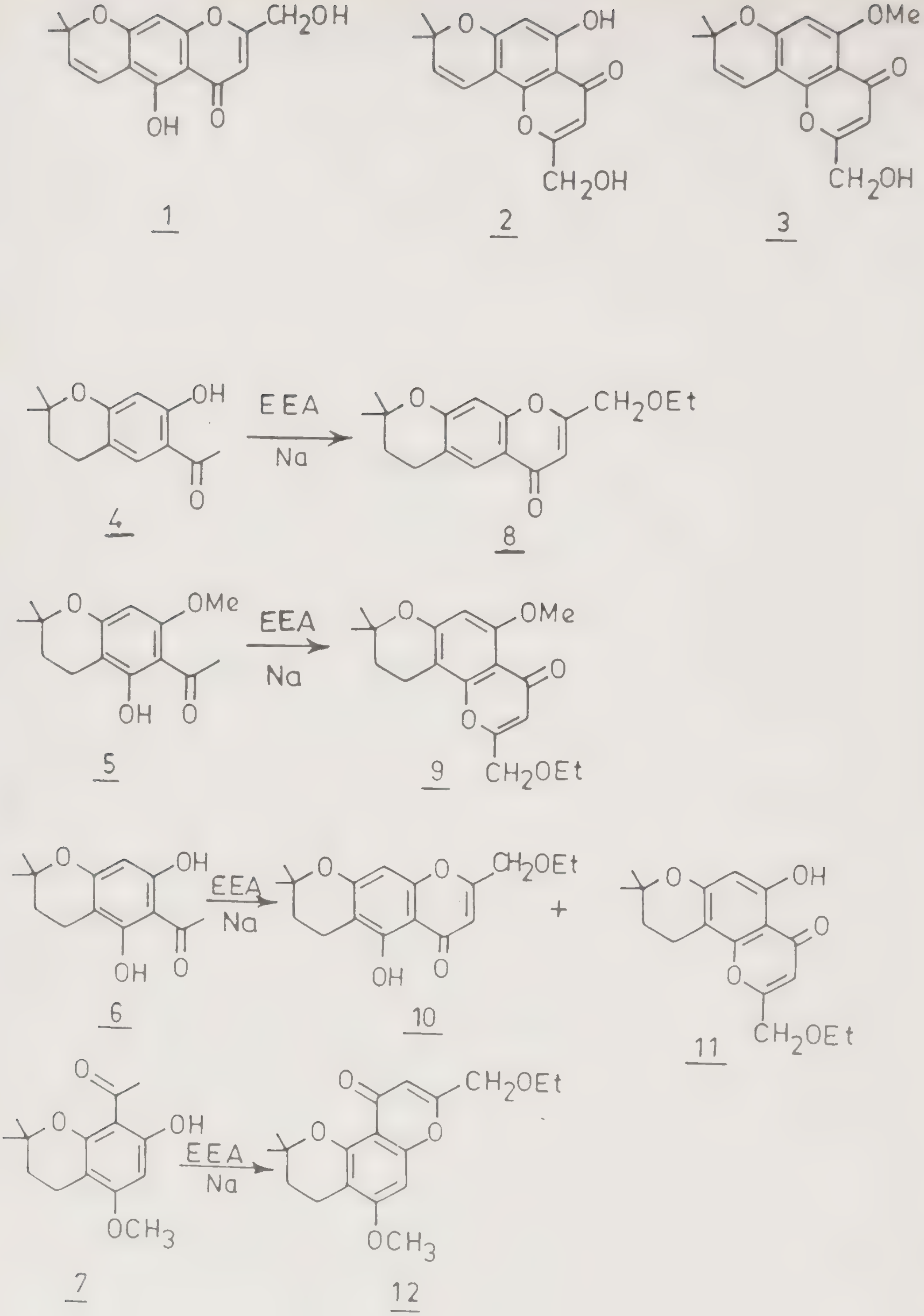
Among the natural pyranochromones, 2-methylchromones are of more common occurrence than 2-hydroxy-methylchromones. Greveichromenol (1), ptaerochromenol (2) and ptaerochromenol methyl ether (3) belong to the latter group. Greveichromenol has been isolated from the heartwood of the Madagascan tree Cedrelopsis grevei /1/, ptaerochromenol has been isolated from the heartwood of Ptaeroxylon obliqum /2/, while ptaerochromenol methyl ether has been reported in the stems of **Cneorum** tricoccum /3/. The synthesis of these compounds is presented in this paper.

We have earlier reported /4,5/ the synthesis of several natural 2-methyl-pyranochromones like spathelia chromene, alloptaeroxylin and its methyl ether. Since alkoxy-chromones have been screened as insecticides /6,7/, fish poisons /6/ and haemostatic agents /8,9/we undertook this work of synthesising 2-alkoxy-methylchromones. At the same time these compounds are the ideal synthons for subsequent conversion to 2-hydroxy-methylchromones.

The products of Claisen condensation of acetyl-chromans (4), (5), (6) and (7) with ethoxyethyl acetate (EEA) and sodium are illustrated (Scheme 1). The PMR spectra of the products showed the typical quartet for $-OCH_2CH_3$ at δ 3.64 and triplet at δ 1.27 for $-OCH_2\underline{CH_3}$ (Figs. 1,2 and 3). Chroman (6) offers two sites for cyclisation and hence a mixture of products (10) and (11) can be expected to be formed. On carrying out the reaction of chroman (6) with EEA and sodium,



SCHEME 1

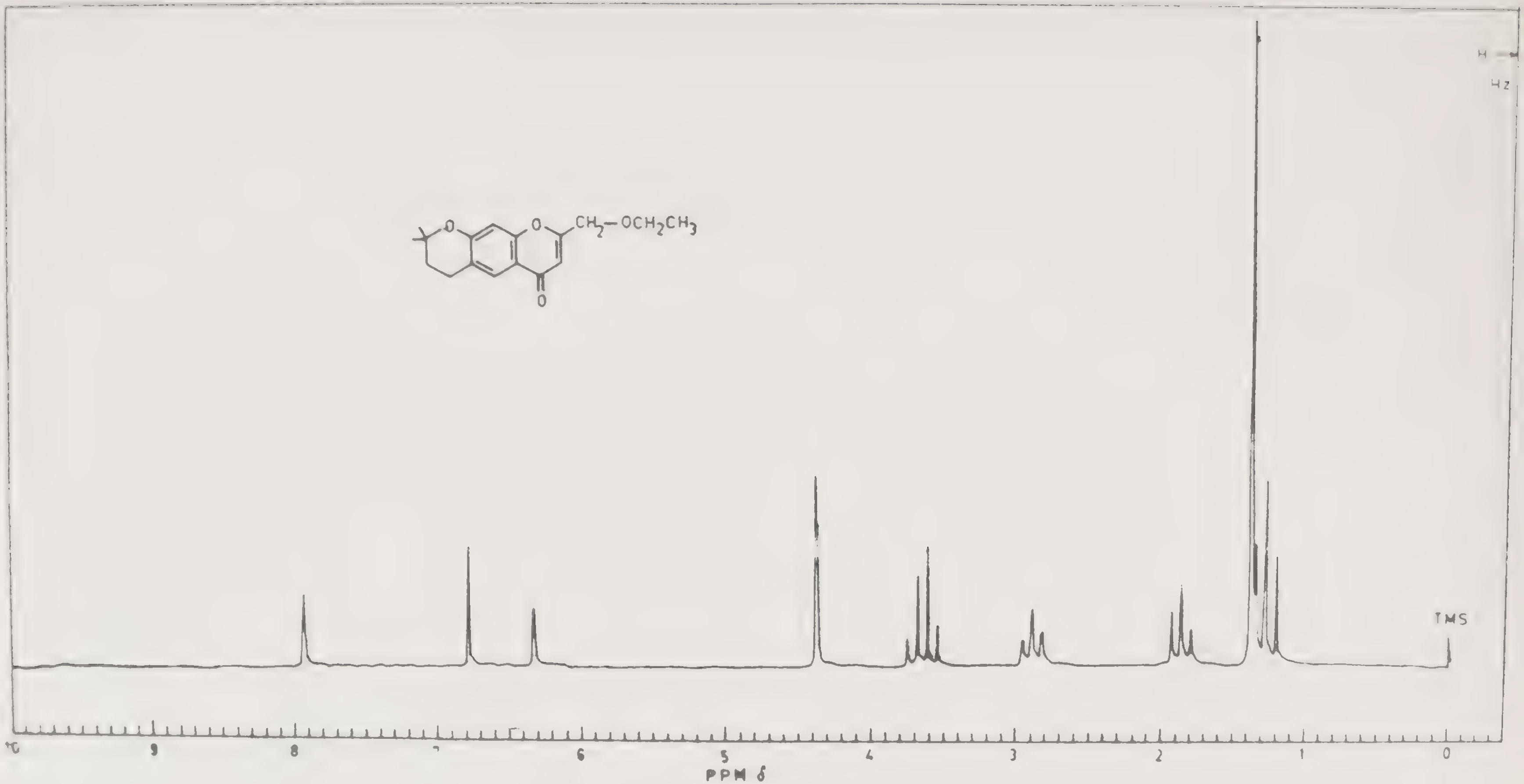


Fig.1 PMR Spectrum of 8

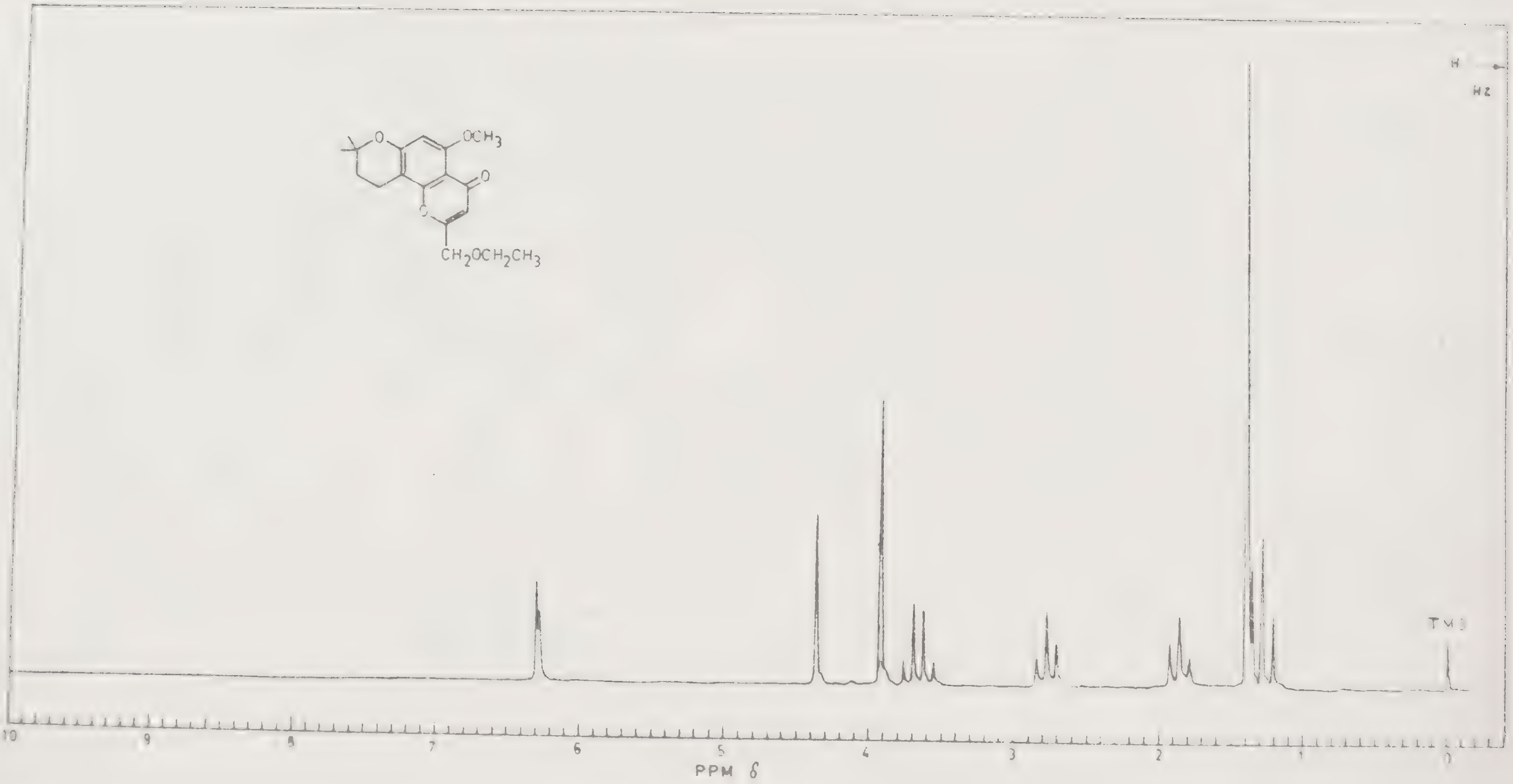


Fig.2 PMR Spectrum of 9

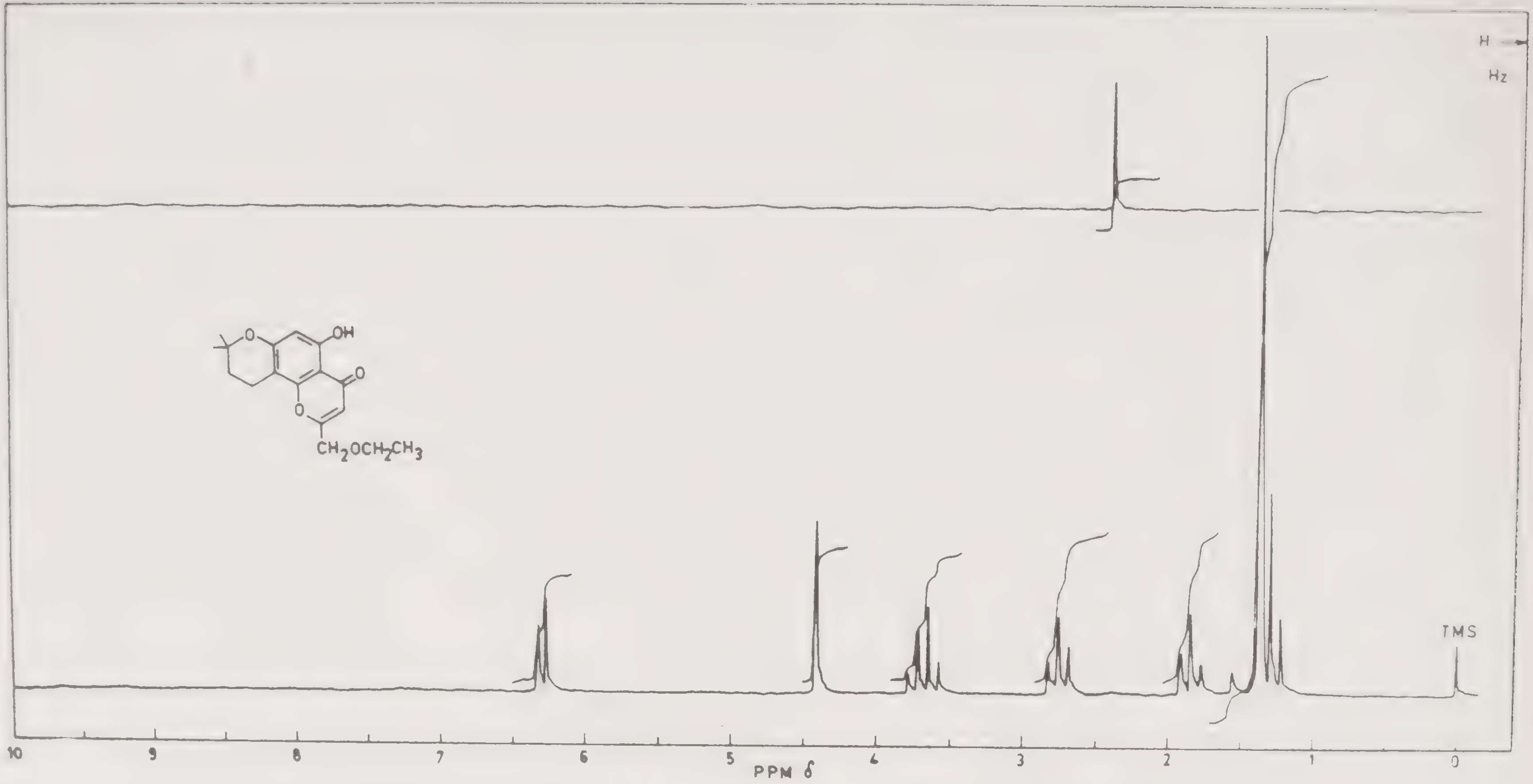


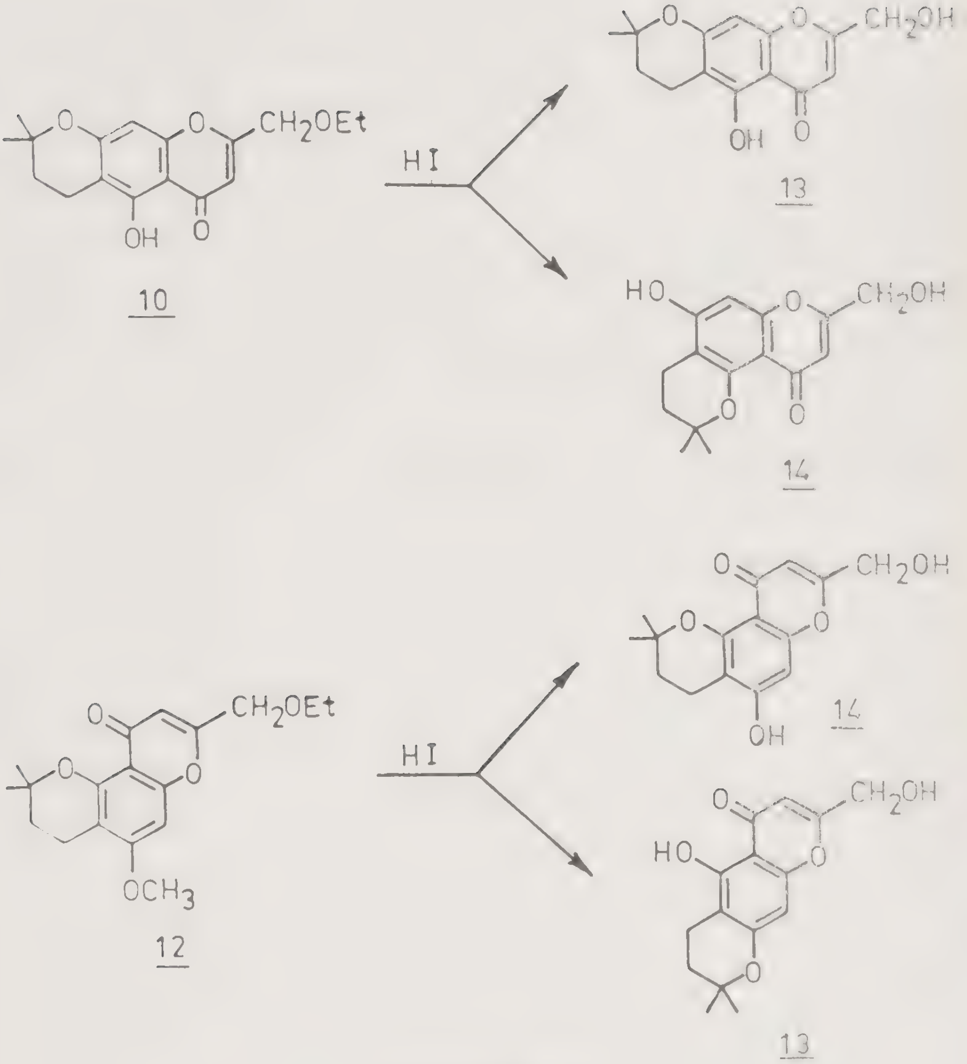
Fig. 3 PMR Spectrum of 11

two products, A and B, were obtained and these were separated by chromatography over silica gel. The chelated hydroxyl protons in A and B appeared around δ 12.4-12.8 in the PMR spectrum (Fig. 3). The products were methylated in acetone using $(CH_3)_2SO_4$ and K_2CO_3. One of these was found to be identical with (9) obtained from chroman (5). Since B gave (9), it was assigned structure (11). A was identified as the linear isomer (10).

In order to get the 2-hydroxy-methylchromones, deethylation was carried out with $AlCl_3/CH_3CN$ using (8) as model compound. Fuji et al. /9/ have reported selective dealkylation of aliphalic methoxyl groups in 2-methoxy-methyl-naphthalene derivatives with $AlCl_3/CH_3CN/NaI$ in the cold. However, treatment of (8) with this reagent in the cold as well as under refluxing conditions (4 h) did not bring about deethylation. In the case of (9) demethylation occurred but deethylation **did** not. The product obtained was identical with (11).

Since deethylation did not occur with $AlCl_3/CH_3CN$, HI was used to bring about this conversion; (9) and (11) on treatment with HI gave dihydroptaerochromenol. Benzylic bromination using NBS/CCl_4, followed by dehydrobromination with pyridine gave ptaerochromenol. Methylation gave ptaerochromenol methyl ether. In case of (10) and (12) in addition to the deethylated products, rearranged products could also be formed (Scheme 2). However, (10) on treatment with HI gave only one major product which was identified as dihydrogreveichromenol. This was subsequently converted to greveichromenol.

It was observed that when (10) and (11) were treated with NBS/CCl_4 followed by pyridine, greveichromenol and ptaerochromenol were obtained. This shows that both dehydrogenation and deethylation have occurred. Pyridine hydrochloride is known to bring about dealkylation and here probably the pyridine hydrobromide brings about deethylation. This is an interesting observation since both dehydrogenation


CH2OEt
OH
10
HI
CH2OH
OH
13
HO
CH2OH
14
CH2OEt
OCH3
12
HI
CH2OH
OH
14
HO
CH2OH
13

SCHEME 2

and dealkylation can be brought about during dehydrogenation with NBS and pyridine.

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NATURAL XANTHONES AND THEIR GLYCOSIDES

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Xanthones are a rapidly expanding group of natural oxygen heterocycles. Their structure is related to that of flavonoids and also their chromatographic behaviour is similar.

In a review [1] published in 1961 only eleven naturally occurring xanthones were mentioned. During the last twentyfour years, however, nearly one hundred and fifty xanthones have been isolated from fungi and higher plants [2,3].

The reason for a growing interest in these compounds is their biological activity, especially antidepressant action by monoamine oxidase inhibition and their tubercolostatic effect.

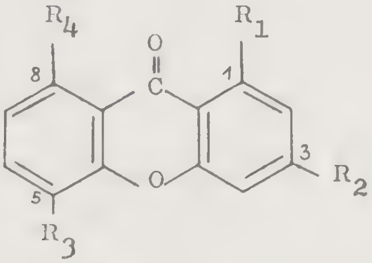
Twenty years ago only four xanthone glycosides were known: mangiferin, a C-glucoside, and three O-glycosides. Today, however, there are more than 30 xanthone glycosides isolated from different plants. As sugar components mono and disaccharides have been identified. It is worthy of comment that β-D-glucose is the only monosaccharide encountered so far whereas in the flavonoid group many other sugars occur. Also, only two kinds of disaccharides have been found in xanthone glycosides. A limited number of bis-glycosides and one acylated xanthone glycoside are also known.

The aglycones are tri-, tetra-, or pentaoxygenated. The unsubstituted or substituted 1,3-hydroxylation pattern is invariably present in each natural compound, and this is combined with substitution in the 4-, 5-, 7- or 8-positions. Most common are xanthone glycosides with tetraoxygenated pattern.

The various positions in which sugars are attached to the xanthone nucleus are noteworthy: of the 24 glycosides of known structure 13 xanthones carry the sugar in position 1 and four in position 8. This preference for positions 1 and 8 is surprising in view of the vicinity of the carbonyl function; a corresponding position in flavonoid compounds (position C-5) is rarely glycosylated. Our synthetic studies may give some explanation for the relative reactivity of the positions adjacent to the carbonyl in xanthones.

The first and until now the only synthesis of some naturally occurring xanthone-O-glycosides was carried out by Wagner [4] and co-workers.

We decided to synthesize some glycosides of a tetrasubstituted xanthone. 1,3,5,8-Tetrahydroxyxanthone (1) (desmethylbellidifolin) has many naturally occurring O-methyl derivatives and five monoglucosides [3]. In all cases the xanthone skeleton carries the carbohydrate unit in position 1 or 8.



	R_1	R_2	R_3	R_4	
1	OH	OH	OH	OH	desmethylbellidifolin
2	OGluc	OH	OH	OH	desmethylbellidifolin-1-O-glucoside
3	OGluc	OMe	OH	OH	
4	OGluc	OMe	OMe	OH	methylbellidifolin-1-O-glucoside
5	OH	OH	OH	OGluc	desmethylbellidifolin-8-O-glucoside
6	OH	OMe	OH	OGluc	
7	OH	OMe	OH	OH	
8	OBz	OMe	H	OH	
9	OH	OMe	OH	OMe	

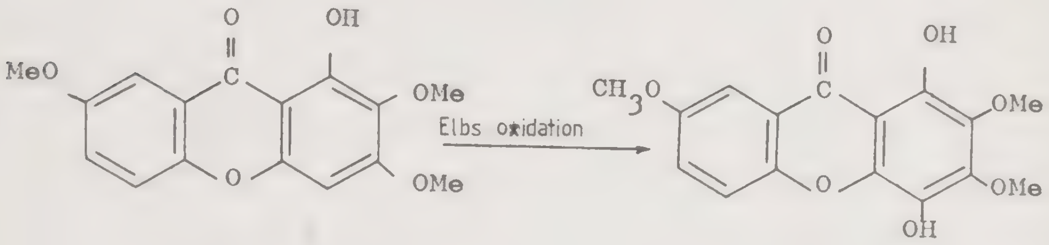
In a synthesis of desmethylbellidifolin glucosides we followed a procedure used before in flavonoid chemistry. A method was devised for the selective glycosidation, in which we utilized the differences in the acidity and reactivity of the different hydroxyl groups using partially benzylated, benzoylated or methylated compounds as aglycons.

Synthesis of the aglycons

First we had to synthesize a suitable derivative of desmethylbellidifolin. Our first plan was to form the para dihydroxy function by Elbs oxidation. We synthesized 1,8-dihydroxy-3-methoxyxanthone (7) which was cautiously benzylated. Interestingly, only the C-1 hydroxy group was benzylated to give 8 in quantitative yield. The formation of the 8-benzyl ether was not observed. The structure of 8 was proved by methylation and debenzylation which resulted in the known 3,8-dimethoxy-1-hydroxyxanthone (9) [5]. Elbs oxidation of this compound was unsuccessful because of the alkali sensitivity of the product.

Markham [6] described the potassium persulfate oxidation of 1,3,8-trihydroxyxanthone on microscale, but in our hands this method failed, too.

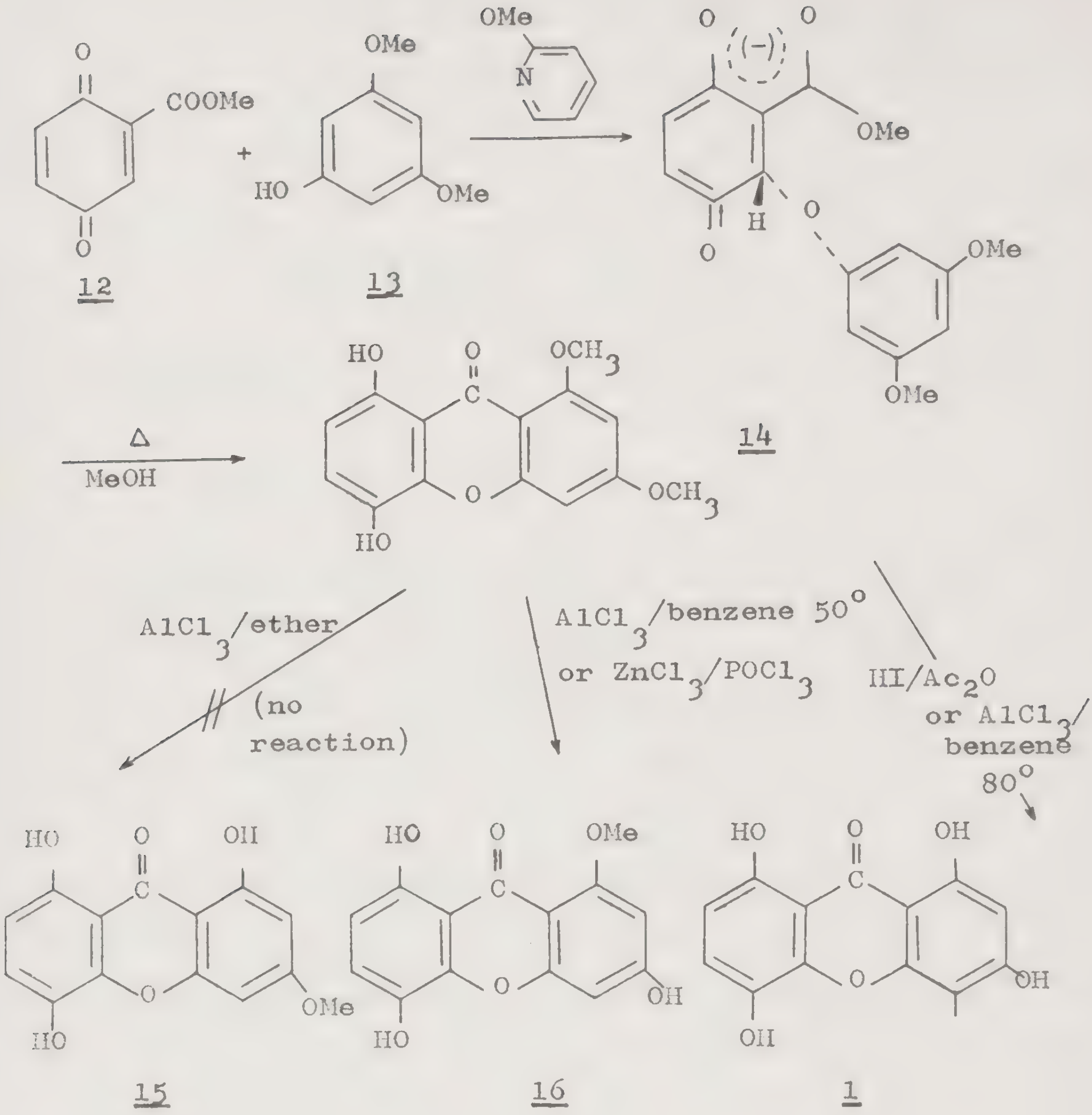
The success of Elbs oxidation seems to depend on the oxidation pattern of the phenol. Thus we successfully oxidized 1-hydroxy-2,3,7-trimethoxyxanthone (10) to 1,4-dihydroxy-2,3,7-trimethoxyxanthone (11). It has to be mentioned that our synthetic product was different from a naturally occurring compound isolated by Ghosal and co-workers [7], for which the same structure was claimed.



10 11

Coming back to the synthesis of the 1,3,5,8-tetraoxygenated xanthones, we decided to try the method of Müller et al. [8], the nucleophilic addition of phenols to alkoxycarbonyl-p-benzoquinones, followed by reduction, methylation, hydrolysis and finally cyclization, or by the oxidative ring closure of o-hydroxybenzoylhydroquinones. We successfully extended the scope of this reaction to our case. The addition of phloroglucinol dimethyl ether (13) to 2-methoxycarbonyl-1,4-benzoquinone (12), using 2-methoxypyridine as basic catalyst, resulted via substituted ether in the required p-disubstituted xanthone. The intermediate diphenyl ether was not isolated, because under the given reaction conditions it cyclized directly to the desired 1,3-dimethoxy-5,8-dihydroxyxanthone (14). The structure of 14 was proved by ^1H- and ^{13}C-NMR spectra.

Our next task was to transform 14 into 3-methoxy-1,5,8-trihydroxyxanthone (15), i.e. to bellidifolin [9,6,10].



It was well known from the chemistry of flavonoids and xanthones that demethylation under controlled conditions preferentially affects the methoxy group adjacent to a carbonyl group. Demethylation of 14 gave, however, a surprising result: using $AlCl_3$ in ether or acetonitrile the starting material remained unchanged. Demethylation with $AlCl_3$ in benzene at 50 °C, with pyridine hydrochloride at 140 °C or with $ZnCl_2$ and phosphorus oxychloride at 60 °C gave, in turn, a monomethoxyxanthone which was different from bellidifolin. ^{1}H-NMR showed the presence of only one chelated hydroxyl group and the ^{13}C-NMR spectra did not show the signals characteristic of a xanthone in which the 1-position was free and the 3-position substituted [11]. So the only possibility remained that our compound was 1-methoxy-3,5,8-trihydroxyxanthone (16). Later we will show another example of the special reactivity of position 1 in xanthones of this type.

Demethylation of 14 by HI in acetic anhydride or by $AlCl_3$ in benzene at 80 °C furnished 1,3,5,8-tetrahydroxyxanthone (1) [10].

In the possession of this compound experiments were made for its selective methylation, benzylation and debenzoylation. We have successfully used these reactions in the selective synthesis of flavonoid glycosides.

Benzylation of 1 with 3 moles of benzyl chloride gave three products, 1,3-dibenzyloxy-5,8-dihydroxy- (17), 3-benzyloxy-1,5,8-trihydroxy- (18) and, as the main product, 5-benzyloxy-1,3,8-trihydroxyxanthone (19). The structures of these compounds were proved by their ^{1}H-NMR spectra and by conversions to derivatives.

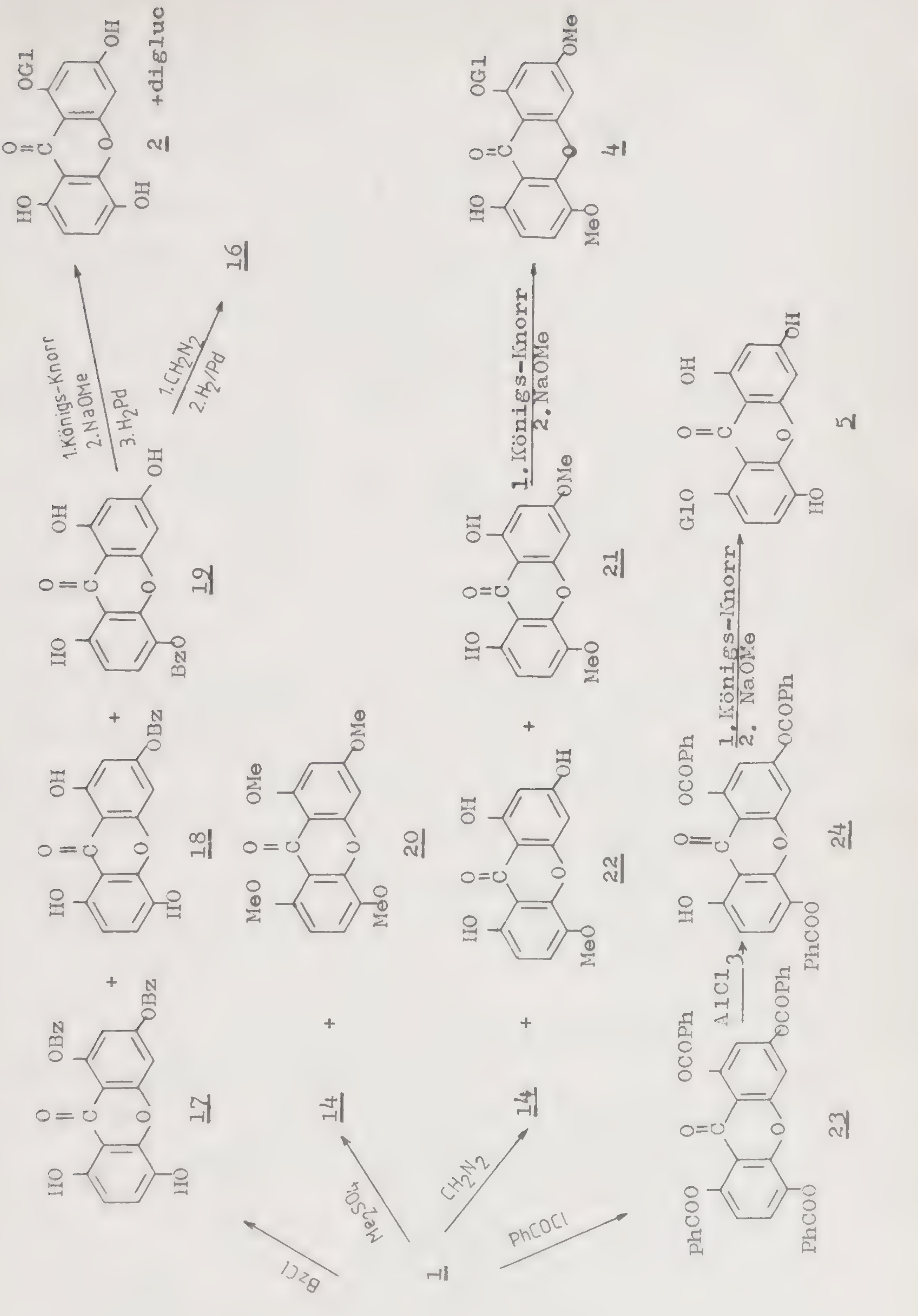
Methylation of the 5-benzyloxyxanthone (19) with 2 moles of dimethyl sulfate and subsequent debenzylation gave the known

1,3-dimethoxy-5,8-dihydroxyxanthone (14). It was interesting that the methylation of 19 with diazomethane in ether and debenzylation gave the 1-methyl ether (16) of the parent xanthone (1).

Methylation of the tetrahydroxanthone (1) with dimethyl sulfate in acetone furnished the tetramethoxyxanthone (20) as the main product; the formation of some 1,3-dimethoxyxanthone (14) was also observed. On the other hand, the methylation of 1 with diazomethane in chloroform at room temperature yielded three products: 3,5-dimethoxy-1,8-dihydroxy- (21), 1,3-dimethoxy-5,8-dihydroxy- (14) and 5-methoxy-1,3,8-trihydroxyxanthone (22) [12].

Thereafter we turned our attention to the selective debenzoylation of 1,3-dimethoxy-5,8-dibenzoyloxy- and 1,3,5,8-tetrabenzoyloxyxanthone (23). When these compounds were treated with aluminium chloride in ether, the C-8 hydroxyl group was liberated. Thus we obtained agluoons with a single free hydroxyl group at C-8 (24).

The experiments presented here did not give a clear picture about the reactivity order of the various hydroxyl groups in the tetrahydroxyxanthone 1. Nevertheless the high reactivity of the hydroxyls at C-5, C-1 was a constant feature and was utilized in the synthesis of methylbellidifolin-1- (4) [13], desmethylbellidifolin-1- (2) [10,14] and -8- (5) [15]-glucosides.


1
BzCl
Me2SO4
CH2N2
PhCOCl
17
18
19
14
20
14
22
21
23
24
1.Königs-Knorr
2.NaOMe
3.H2Pd
2 +digluc
1.CH2N2
2.H2/Pd
16
1.Königs-Knorr
2.NaOMe
4
AlCl3
1.Königs-Knorr
2.NaOMe
5
HO
OBz
OH
OG1
OMe
MeO
BzO
G1O
PhCOO
OCOPh

Synthesis of the glucosides

For the synthesis of desmethylbellidifolin-1-0-glucoside (2) the 5-benzyl compound (19), for methylbellidifolin-1-0-glucoside (4) the 3,5-dimethoxyxanthone (21) seemed to be suitable aglucons. On coupling of 21 with α-acetobromoglucose according to Königs and Knorr, a monoglucoside fraction was isolated as the main product which could only be the desired 1-glucoside (4). In the case of 18 the required monoglucoside (2) was accompained by the 1,3-diglucoside. The physical constants of our synthetic compounds agreed with those reported for the natural products.
Unfortunately, natural samples were not available.

For the synthesis of the desmethylbellidifolin-8-glucoside (5) the above mentioned 8-hydroxy-1,3,5-tribenzoyloxyxanthone (24) seemed to be an ideal aglycon. Coupling of this compound with α-acetobromoglucose gave in a prolonged reaction, and in a very poor yield, the acylated 8-0-glucoside; subsequent saponification yielded desmethylbellidifolin-8-0-glucoside (5).

The synthesis of the 3-methoxy-1,5,8-trihydroxyxanthone-glucosides could not be accomplished because the rational synthesis of the aglycon yet has not been solved.

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KINETIC INVESTIGATIONS OF THE ELIMINATION REACTION OF 3-BROMOFLAVANONES

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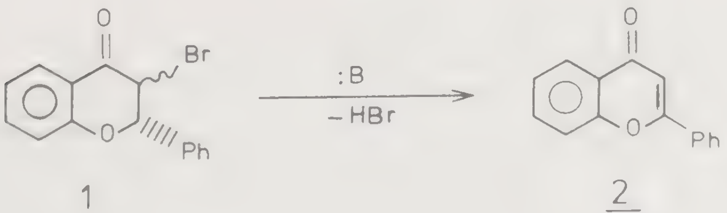
INTRODUCTION

As a part of our studies on the reaction of 3-alkyl- and -arylsufonyloxyflavanones with nucleophiles we decided to investigate the mechanism of HBr elimination of cis- and trans-3-bromoflavanones (1), which may provide a suitable model for the examination of more complex reactions of 3-mesyloxyflavanones /1-4/.

Dehydrobromination of 1, leading to the corresponding flavone 2, has been long known as a convenient method for the preparation of flavones from flavanones. ß-Elimination was reported to proceed in the presence of various bases /5-15/ or upon heating /11, 16/, and even on standing in a dipolar aprotic solvent (DMSO) at room temperature /15/, as well as in the course of bromination of flavanones /10/. At the same time, no systematic studies on the exact mechanism of the elimination reaction of the isomres of 1 have been hitherto reported.



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2

KINETIC MEASUREMENTS

Our preliminary investigations confirmed the earlier observation /12/ that the rate of the elimination reaction of cis- and trans-1 is nearly the same, though on the basis of stereochemical considerations cis-1 is expected to react faster.

We found that this is the situation in the presence of strong bases (alkali, amines), but with weak bases (e.g. halide ions) a sharp difference can be observed between the reactivity of the two isomers, and cis-1 was shown to be much more unstable (Table 1).

Table 1. Data of the conversion of cis-3-bromoflavanone into flavone in different systems
($c_{3-Br} = 10^{-4}$ mol.dm^{-3}; $c_{salt} = 10^{-1}$ mol.dm^{-3})

Salt	Solvent		
	MeCN	DMF	N-Me-pyrrolidone
None	stable	slow decomp.	350 min: 60%
$Et_4N^+Cl^-$	~70 min: 100%*	~50 min: 100%	-
anh. LiCl	ns	~12 min: 100%	< 2 min: 100%
NaBr	ns	~50 min: 100%	-
KNO_3	—	18 min: < 2% 195 min: 15%	-

ns: solubility less than 10^{-1}mol.dm^{-3}
*: similar data of trans-3-bromoflavanone: 40 min: < 2%
3 days: 27%

The variance of the reactivity clearly shows that the elimination reaction of 3-bromoflavanones proceeds in a complex way.

The typical kinetic curves, obtained by means of UV spectrophotometric method[+], are shown in Figs. 1 and 2. The curves recorded at 315 nm are very similar to those presented below.

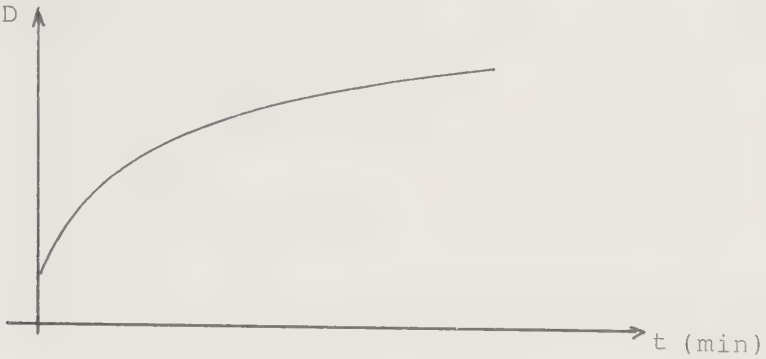

Fig. 1. Kinetic curve of cis-1 (λ= 290 nm, c_{CHA}= 3×10^{-3} mol. dm^{-3}, T=29.9°C)

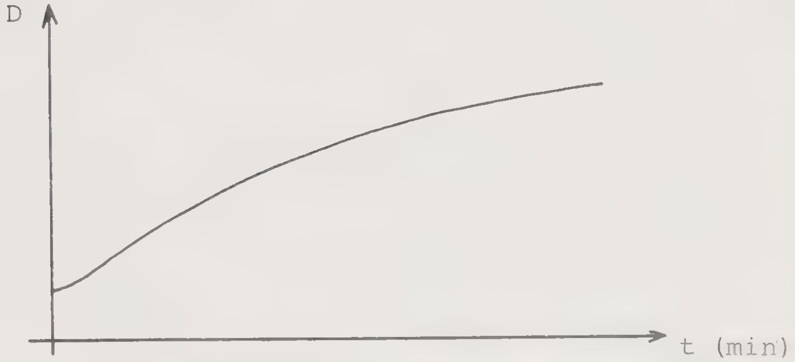

Fig. 2. Kinetic curve of trans-1 (λ= 290 nm, c_{CHA}= =3×10^{-3} mol. dm^{-3}, T=30°C)

[+] The reactions were performed in DMF solution using cyclohexylamine (6×10^{-4}-1×10^{-2} mol.dm^{-3}) as the base. Ionic strength was adjusted to $I=10^{-1}$ with potassium nitrate. The kinetic curves were recorded at 1×10^{-4} mol.dm^{-3} initial concentration of 1 at wavelengths 290 and 315 nm and temperatures 25, 30, 35 and 40°C. The measurements were accomplished by using at least six different amine concentrations at each wavelength and temperature.

Comparison of the kinetic curves of the two isomers demonstrates a difference only in the initial section, but they are similar for the whole reaction. At the same time, the kinetic curve of trans-1 also indicates a complex process.

As the first approach for the analysis of the kinetic curves a three-parameter equation (Eq. 1) was applied

$$D = p_1 e^{p2t} + p_3 \tag{1}$$

being alive to the fact that in the case of the trans isomer this relationship expresses only a gross description and for an exact analysis at least five parameters (introduction of an additional exponential term) are necessary.

Comparison of the results of nonlinear regression calculations* demonstrated that the p_1 and p_3 parameters are constants, whereas the $k_a = -p_2$ (the apparent rate constant) depends on the concentration of the amine.

Data revealed that the $k_a = -p_2$ values, calculated from the curves obtained at different wavelengths, are practically identical. (The p_1 and p_3 parameters, as they involve the molar absorption coefficients, are naturally different).

The values of p_1, p_2 and p_3, calculated for the cis and trans isomers from the UV curves recorded under identican conditions (amine concentration wavelength, temperature), are in good accordance. This result gives a quantitative proof for the nearly identical reactivity of the two isomers.

Typical examples for the dependence of the k_a value on the amine concentration is given in Figs. 3 and 4. Similar diagrams were obtained at the other temperatures.

The dependence of the apparent rate constants on the amine concentration proved to be linearizable simultaneously in the forms:

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*: The determination of the parameters was accomplished by using maximal points (with absorbance values less than 1.2) on an R-30 computer with BMD 3RP program /17/, or on a Commodore 64 PC with a nonlinear least-square-method program, working with the Gauss-Newton method.

$$k_a = m\,[CHA] + b \qquad (2)$$

$$\frac{k_a}{CHA} = m\,\frac{1}{[CHA]^2} + b \qquad (3)$$

(see Tables 2 and 3), although the latter gave better fit.

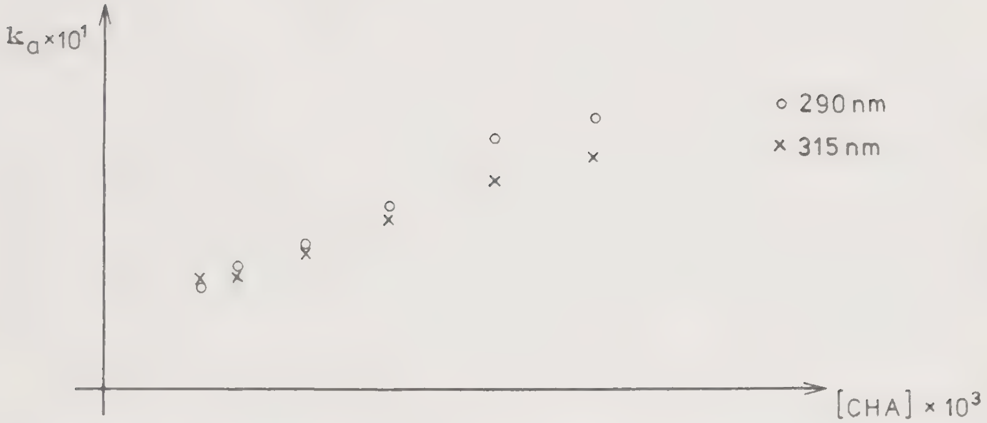


Fig. 3. Dependence of k_a on [CHA] (cis-1, T=29.9 °C)

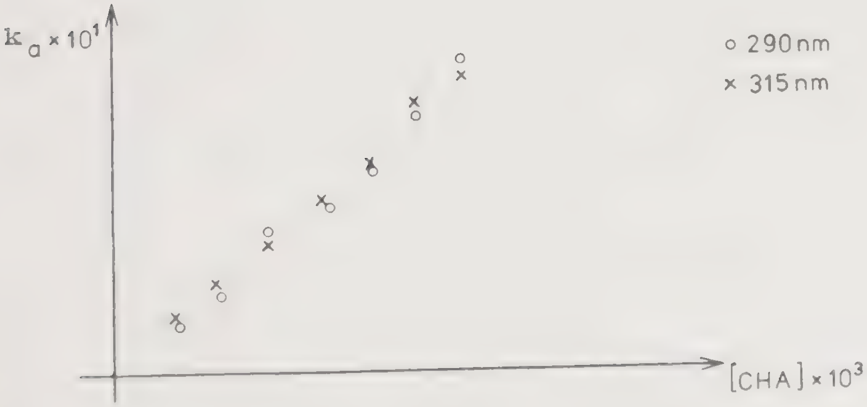


Fig. 4. Depencence of k_a on [CHA] (trans-1, T=30 °C)

Table 2. Parameters of the $k_a = m[CHA] + b$ correlation for cis-1

T(°C)	λ(nm)	m	bx10^2	N*	r^2	t_b
25	290	40.51±3.02	5.724±0.966	6	0.9782	5.93
		44.93±2.52	4.043±0.876	5	0.9906	4.61
	315	38.45±3.22	5.357±1.256	6	0.9726	4.27
		42.49±3.40	3.470±1.441	5	0.9812	2.41
29.9	290	49.99±3.65	4.177±1.110	6	0.9791	3.76
		52.18±4.45	3.324±1.477	5	0.9786	2.25
	315	37.80±1.96	5.551±0.596	6	0.9894	9.32
		40.06±1.24	4.672±0.409	5	0.9972	11.41
35	290	64.91±4.59	8.590±1.044	6	0.9804	8.23
		67.85±4.76	7.681±1.179	5	0.9854	6.51
40.2	290	89.86±8.25	8.368±1.412	6	0.9674	5.93
		95.11±9.00	7.186±1.670	5	0.9738	4.30

*: Values for N = 5 were obtained by neglecting the lowest concentration.

Table 3. Parameters of the $k_a/[CHA] = m/[CHA]^2 + b$ correlation for cis-1

T(°C)	(nm)	m x10^5	b	N	r^2	t_b
25	290	5.764±0.251	51.35±1.08	6	0.9931	47.65
	315	5.906±0.337	46.88±1.43	6	0.9872	32.82
29.9	290	3.541±0.229	57.64±1.93	6	0.9835	29.89
	315	4.477±0.078	49.28±0.63	5	0.9991	78.35
35	290	5.307±0.202	87.58±2.77	6	0.9942	31.62
40.2	290	5.003±0.264	116.00±3.63	6	0.9890	31.92

Owing to the simplification used for the description of the kinetic curve of trans-1, its data do not allow an evaluation based on Eq. (3). Nevertheless, the slope values obtained by Eq. (2) are in good agreement with those received for cis-1.

The data of Table 2 clearly show that the intercept obtained by the analysis of k_a is a low value, but significantly different from zero. This indicates a complex process again.

On the basis of the shape of the kinetic curves, of the numerical values of the p_1-p_3 parameters and of the linearization by means of Eq. (2), the elimination reaction of the 3-bromoflavanone isomers in the presence of strong bases can be described by the following model:

$$\begin{tikzcd}
\text{trans-1} + \text{CHA} \arrow[r, shift left, "k_1"] \arrow[rr, bend right, "k_3"'] & \text{cis-1} \arrow[l, shift left, "k_{-1}"] \arrow[r, "k_2"] & 2
\end{tikzcd}$$

where k_1, $k_{-1} >> k_2$ k_3. This means the existence of a trans $\rightleftarrows$ $\rightleftarrows$ cis isomerization during a pre-equilibrium, and thus most part of the flavone (2) is produced from cis-1.

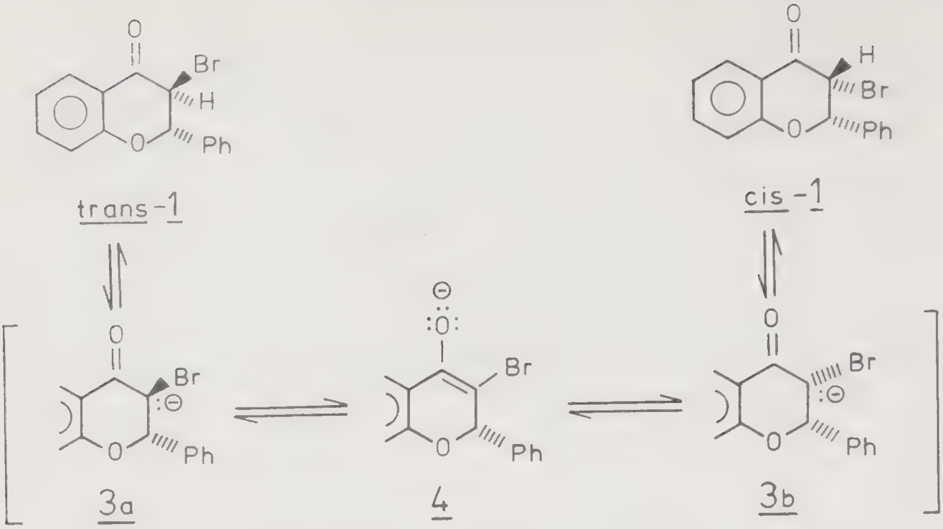
From this model the apparent rate constant of the formation of flavone can be expressed as

$$k_a = \frac{k_3 + k_2K[CHA]}{1 + K[CHA]} \tag{4}$$

where $K = k_1/k_{-1}$. On the basis of the experimentally obtained k_a -[CHA] relations, the foregoing reaction is described by that case of the model where the transformation cis-1 → 2 is a second order reaction (i.e. $k_2 = k_2'[CHA]$), the process trans-1 is of first order, and $K[CHA] >> 1$ (the equilibration is shifted towards the cis isomer). In such a situation both Eqs (5) and (6) are valid,

$$k_a = \frac{k_3}{K} \cdot \frac{1}{[CHA]} + k_2' [CHA] \tag{5}$$

$$\frac{k_a}{[CHA]} = \frac{k_3}{K} \cdot \frac{1}{[CHA]^2} + k_2' \tag{6}$$



The C-3 epimerization observed during the reduction of trans-3-chloroflavanone with sodium borohydride was explained earlier by Weber et al. /18/ by supposing the formation of an enol intermediate of type 4, but no proof for this assumption was given. In the case of steroid α-halogenoketones /19/ the similar epimerization process was exactly shown /20/ to proceed via the enol intermediate. Studies on 2-halogeno-3-methoxy-1,3-diphenylpropane-1-ones have revealed /21/ the epimerization of the halogen atom in the presence of bases through a carbanion intermediate.

For proving the supposed mechanism for the above epimerization, detailed ^{1}H-NMR spectroscopic studies were made. These investigations showed that in the presence of weak bases (pyridine-d_5) in DMSO-d_6-D_2O solution a slow elimination occurred. The reactivity of the two isomers is different (cis-1 completely transforms within three days, whereas 65% of trans-1 remains unchanged), proving the existence of k_2 and k_3 and also the assumption made for their magnitude. Significant epimerization and/or deuteration were not observed.

At room temperature the reaction performed in the presence of strong bases cannot be monitored by the NMR technique. In the "frozen" spectrum (reaction mixture containing D_2O and less than one equivalent of CHA) the signals of both the cis- and trans isomers appear, deuteration to a large extent at position 3 is observed, and the produced flavone is also highly deuterated. With an excess of the amine both isomers gave the same final product: compound 2 deuterated at H-3 to an extent of 80-85%.

These results unequivocally support the presumed reaction mechanism, and the absence of deuteration at position 2 excludes an ElcB mechanism. On the basis of the second-order reaction kinetics and the relatively high negative value of the activation entropy (Table 4), a synchronic E2 mechanism is presumed for the elimination reaction. This is in accordance with the mechanism supposed earlier for the elimination reaction of some tertiary α-halogenoketones /22/.

Further studies for a more exact description of the process is in progress.

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REACTION OF 3-MESYLOXYFLAVANONES WITH S-NUCLEOPHILES

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Recently we have reported the reaction of 3-mesyloxyflavanones (1) with thiocyanate and thioacetate ions leading exclusively to 3-thiocyanato- and 3-thioacetatoflavanones by nucleophilic substitution reactions /1/. The high reactivity of the mesyl ester 1 towards S-nucleophiles affords a good opportunity to synthetize novel, hitherto unknown flavanones carrying a sulfur atom at position 3. We now report further results in this field by using thiols or thiolates as the nucleophiles.

When trans-3-mesyloxyflavanones (1) were reacted with thiophenol or sodium benzenethiolate under various conditions, 3-phenylthio-4'-R-substituted-flavanones (2) were isolated in moderate-to-good yields, together with the corresponding flavone (3), 2'-hydroxychalcone (4) and diphenyl disulfide (5).

Product-ratio data (summarized in Table 1) reveal that enhancing the polarity of the solvent the displacement reaction accelerates, but also the secondary transformations become faster; furthermore, excess of the nucleophile favours the formation of the by-products. The data show that the reactions are promoted by the phase-transfer catalyst Adogen-464, but not by 18-crown-6.

Good results were achieved with thiophenol and K_2CO_3 in the presence of Adogen-464 (a modification of the method reported by Lissel /2/), but the reaction of 1a with sodium benzenethiolate in dry benzene was found to give better yields and higher purity of the product.

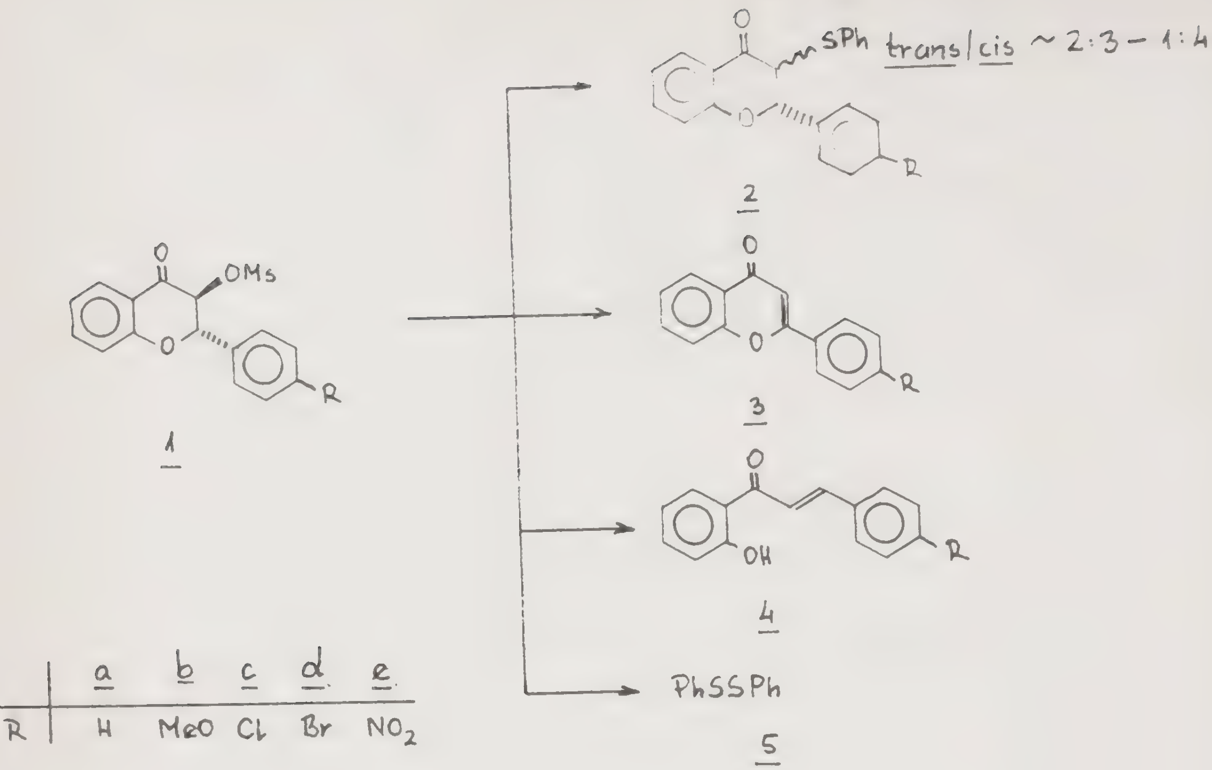


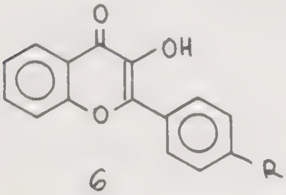
Table 1. Product-ratio data of the reaction of trans-3-mesyloxyflavanone (1a) with PhSX

X	Conditions	PhSX / MsO	Yield (%)					
			1a$^+$	2a	3a	4a	5	6a
H	PhH/K_2CO_3/A-464 46 h	1.39	0	84.7	tr	6.8	19.2	tr
Na	Me_2CO/20 min	1.57	0	30.5	tr	46.9	30.5	0
	/30 min	1.02	1.5	73.3	tr	17.6	9.0	0
	MeCN/35 min	1.02	1.0	77.5	4.3	17.5	6.0	0
	PhH/26 h	1.02	0	90.6	tr	<1.0	1.5	0
	1 h/A-464	1.02	10.2	72.0	tr	4.3	4.5	5.0
	72 h/18-C-6	1.02	30.3	44.6	12.0	3.9	8.2	0

+: Unreacted starting material

tr: traces A-464: Adogen-464 18-C-6: 18-Crown-6

Similar experiences were obtained with the reaction of the 4′-substituted derivatives 1b-e (Table 2), i.e. PhSNa (Method B) reacts slower, but gives better yields. In the phase-transfer procedure (Method A) the secondary reactions leading to the minor products are more considerable, particularly when the molecule carries an electron-attracting substituent at position 4′. In this case 3-hydroxy-4′-R-flavones (6) and 2′-hydroxy-4-R-α-phenylthiodihydrochalcones [1-(2-hydroxyphenyl)-3-(4-R-phenyl)-2-phenylthio-1-propanones] (7) were isolated from the reaction mixture in addition to the compounds mentioned earlier.



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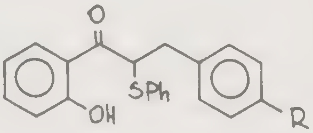

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Table 2. Product-ratio data of the reaction of 3-mesyloxy-4′-R-flavanones (1b-e) with PhSX

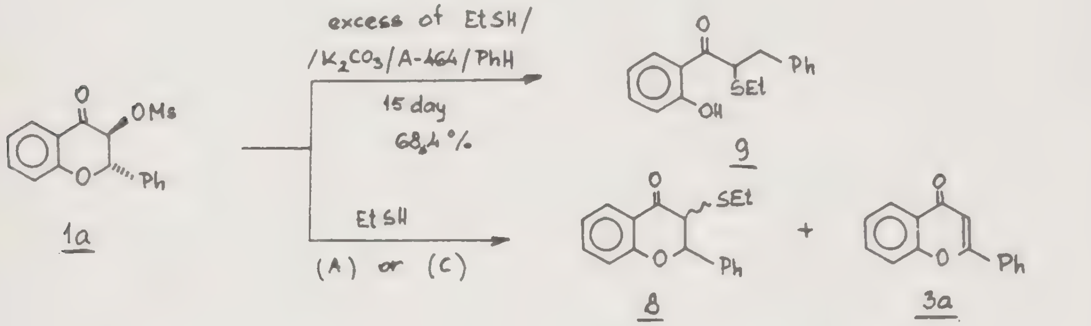
R	Method	Time (h)	Yield (%)						
			1+	2	3	4	5	6	7
MeO	A	90	0	62.6	3.3	5.6	21.7	5.7	0
	B	50	57.3	24.4	9.9	*	7.9	0	0
Cl	A	90	0	53.5	0	14.2	19.5	7.8	*
	B	7.5	6.9	84.1	3.2	2.2	2.9	2.1	0
Br	A	25.5	tr	73.6	0	6.5	25.2	*	8.1
	B	2.5	6.1	85.4	2.0	1.9	3.5	1.3	0
NO_2	A	21.5	0	32.2	9.0	4.1	25.3	11.2	18.5
	B	36.5	11.7	65.4	4.5	2.9	2.8	0	0

+: unreacted starting material *: not isolated tr: traces

Method A: 1.4 eq PhSH/K_2CO_3/Adogen-464/PhH

Method B: 1.02 eq PhSNa/abs. PhH

Reaction of 1a with ethanethiol afforded 3-ethylthioflavanone (8); phase-transfer conditions (Method A) gave better yields than the EtSH/Et_3N/acetone system /3/, where the great quantity of triethylamine promotes the concurrent eliminiation reaction /4/, leading to flavone (2a). 3-Ethylthioflavanone was synthetized earlier in a multi-step process in a lower yield /5/.

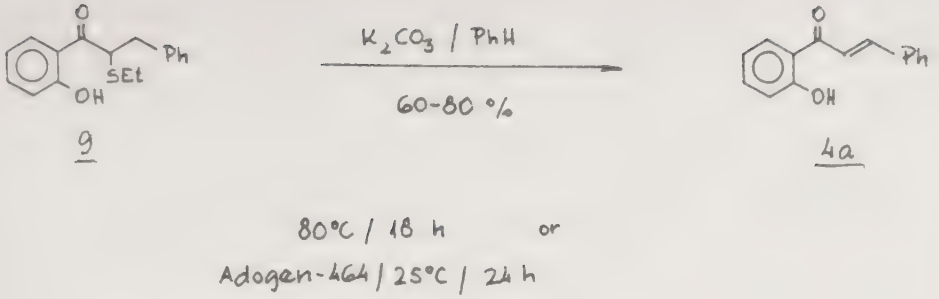


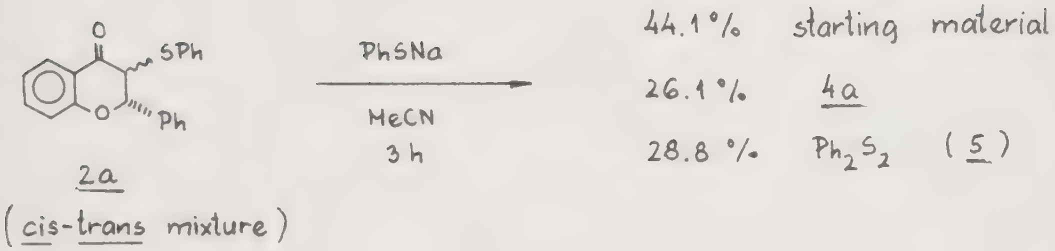
		8	3a
(A):	K_2CO_3/A-464/PhH/48 h	82 %	traces
(C):	Et_3N/Me_2CO/72 h	57.2 %	18.6 %

It is noteworthy that with excess thiol and longer reaction time the initially formed 8 transformed into 2′-hydroxy-α-ethylthiodihydrochalcone (9). The formation of dihydrochalcones 7 and 9 may be explained by an oxidation-reduction process, taking place presumably via 2′-hydroxy-α-SR-chalcones. The reaction is similar to the disproportionation observed in the case of 3-thioacetatoflavanones /1/, but here the oxidized product is the disulfide (e.g. 5) formed from the thiol.

Dihydrochalcones 7 must be considered as the precursors of 2′-hydroxychalcones (4), in agreement with the product-ratio data indicated in Table 2. This is supported by the observation that 2′-hydroxy-α-ethylthiodihydrochalcone (9) transforms into 2′-hydroxychalcone (4a) in the presence of base.

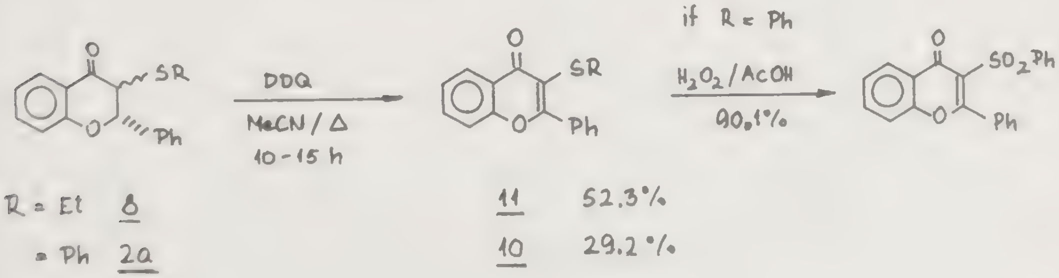
The reaction of 3-phenylthioflavanone (2a) with sodium benzenethiolate, leading to 2′-hydroxychalcone (4a), gives further evidence for the proposed sequence.



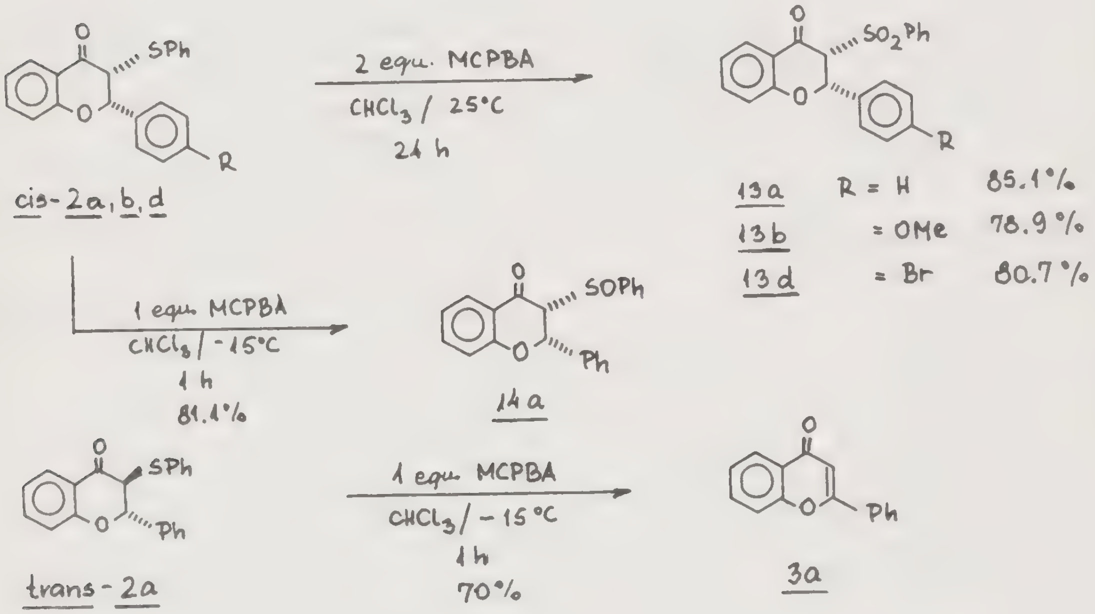


3-Phenylthioflavanone (2a) and 3-ethylthioflavanone (8) were dehydrogenated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to 3-phenylthioflavone (10) and 3-ethylthioflavone (11), respectively. 3-Phenylthioflavone was found to be oxidizable into 3-phenylsulfonylflavone (12) in excellent yield.

These transformations have practical importance providing a new route for flavones claimed to possess biological activity. 3-Methylthioflavones have been reported as CNS-depressants /6/, whereas for 3-phenylsulfonylflavones and -chromones antiallergic effect /7/ was mentioned.



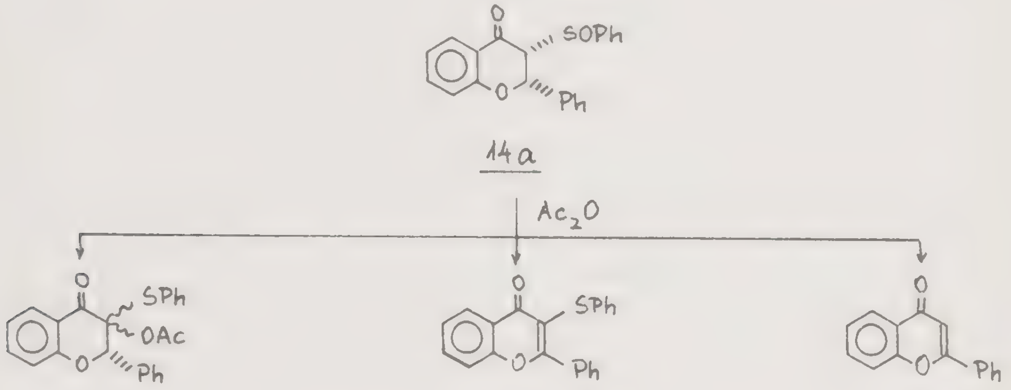
The 2a isomers showed completely different behaviour on oxidation. When cis-2a was oxidized with m-chloroperbenzoic acid (MCPBA) at room temperature, cis-3-phenylsulfonylflavanone (13a) was obtained. At lower temperature and with short reaction time cis-3-phenylsulfinylflavanone (14a) was prepared, but similar reaction of trans-2a gave flavone (3a) as the sole product (verified by ^{1}H-NMR measurement) instead of the expected trans-sulfine (or sulfone).



This different behaviour can be explained most plausibly by stereochemical reasons; the trans-sulfine (or sulfone) becomes unstable owing to the trans-diequatorial arrangement of the two bulky substituents, resulting in considerable strain.

It is noteworthy that 3-methylsulfinylflavanones (prepared from the corresponding acetophenones) have been reported as CNS-depressants /8/.

Low stability of cis-3-phenylsulfinylflavanone (14a) was observed as this compound was found to transform into 3a on standing or on attempted recrystallization. A similar elimination reaction of α-phenylsulfinyl-ketones is known in the literature /9/. When 14a was reacted with acetic anhydride, 3-acetoxy-3-phenylthioflavanone (15), 3-phenylthioflavone (10) and flavone (3a) were produced. The presence of acid catalyst seems to be necessary for the acetylation process, but at the same time it promotes the subsequent AcOH eliminiation. A similar reaction was observed in the case of cis-3-methylsulfinylflavanones /10/.



	15	10	3a
in the presence of conc. H_2SO_4, 24 h	6.7 %	56.6 %	7.5 %
without H_2SO_4, 95 h	16.1 %	15.6 %	73.7 %

In sum, the reaction of 3-mesyloxyflavanones with thiols and thiolates offers a convenient method for the synthesis of 3-alkyl- or 3-arylthioflavanones and their derivatives. Further results, achieved in this field, will be published in the near future.

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QUANTUM CHEMICAL STUDIES ON THE UV SPECTRA OF PARENT FLAVONOIDS

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Introduction

Ultraviolet spectrophotometry (UV) is still one of the most frequently applied methods in flavonoid chemistry for both instrumental analysis and structural investigation [1-2].

Due to their conjugated unsaturated system, flavonoid compounds possess characteristic UV absorptions which have been studied by several research groups [1-9]. However, the already published considerations - based on the resonance theory - give only qualitative and unsophisticated explanation of the structural features.

Nowadays, modern quantum chemistry offers a more accurate and reliable interpretation of the UV spectral characteristics, although the presently available methods are of not equal ability. The earlier systems are based on the $\sigma-\pi$ electron separation theory, regarding only the π electrons and substituting the effect of the σ electrons by the potential due to the rigid atomic core (e.g. the PPP=Pariser-Parr-Pople or GMS=Goeppert-Mayer-Sklar methods) [10].

These procedures approach well certain properties (e.g. reactivity data, etc.) of a substance belonging to a given series of compounds. At the same time, for proper interpretation of the spectral and reactivity parameters, the effect of all of the electrons must be taken into consideration. The quantum chemical methods enabling such calculations are called "all valence" methods (e.g. CNDO=Complete Neglect of Differential Overlap and INDO=Intermediate Neglect of Differential

Overlap) [10]. In the field of flavonoid compounds only a few "all valence" calculations have been reported [9].

The present work deals with the investigation of the flavonoid parent compounds by examining their UV spectral data by the PPP and CNDO/S quantum chemical methods.

Experimental

The calculations were performed with the R-30 computer of Kossuth Lajos University (Debrecen). The geometric parameters, used for the calculations (standard bond lenghts and bond angles), were taken from the literature [11]. The CNDO/S calculations were performed by applying a modified QCPE-333 program [12], whereas for the PPP computations a self-made program was used.

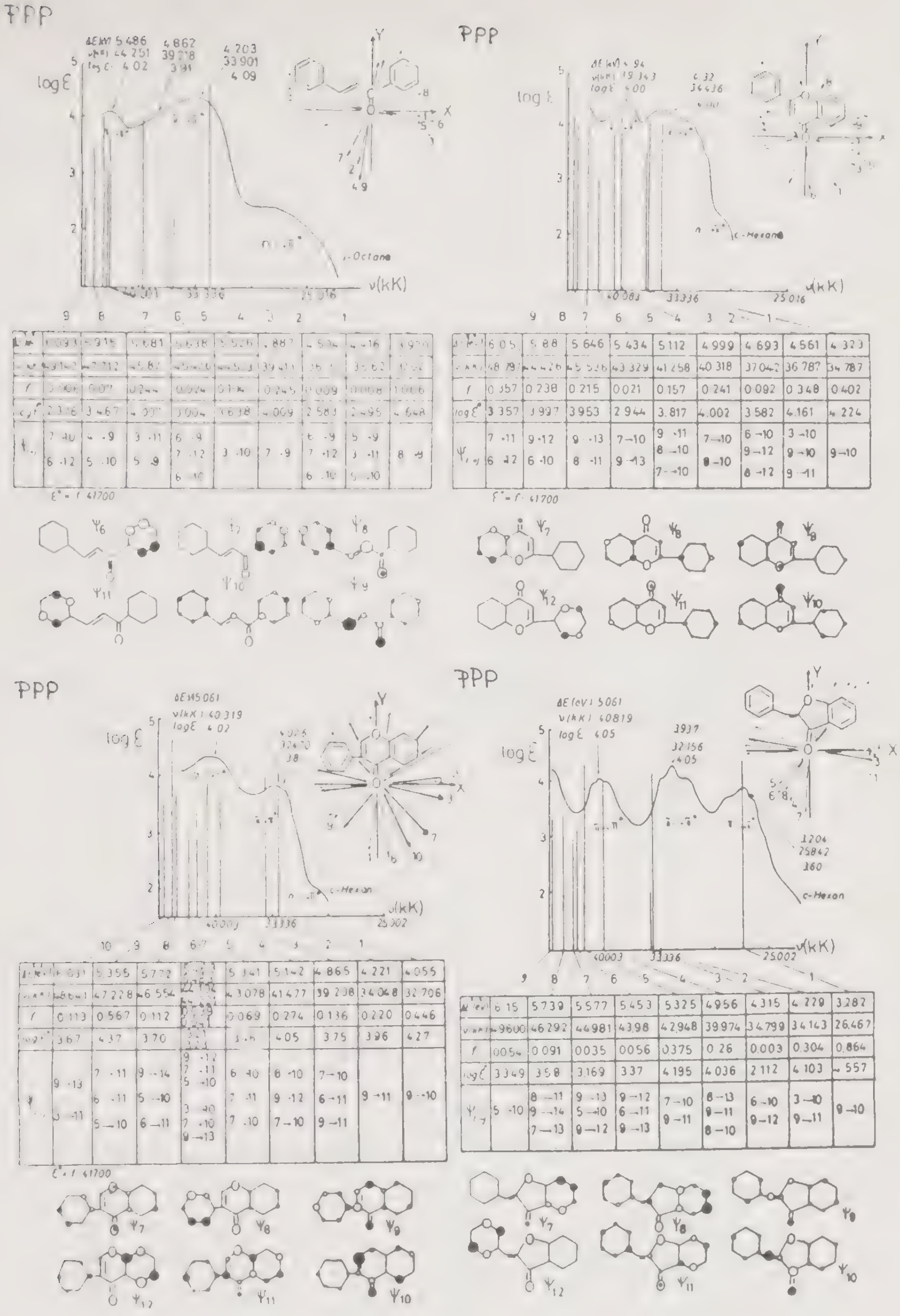
The PPP method was used in SCF MO-LCAO CI form. All of the single configurations are taken into account in the configuration interaction (CI). The one-center coulomb repulsion integrals (γ_{ii}) were approximated according to Pariser and Parr [13] as the difference between the ionization potential [14] and the electron affinity (A_i) of valence states. The two-center repulsion integrals (γ_{ij}) are calculated by the Nishimito-Mataga approximation [15]. The resonance integrals (β_{ij}) are calculated by Flurry's method [16]. The UV absorption spectra were recorded for solutions in ethanol with a Perkin Elmer 554 instrument.

Results and Discussion

A part of the studies was aimed at making comparison between the results obtained by means of the PPP and CNDO/S methods for chromone, flavone, isoflavone, aurone and chalcone.

The data in Figure 1 clearly show that the PPP method describes well the places of the experimentally measured bands, but the intensity relations are insufficiently reproduced.

Figure 1 demonstrates also the participation of the molecular orbital in the formation of the respective UV transition and the results show a complete accordance with earlier



	9	8	7	6	5	4	3	2	1
ΔE (eV)	[illegible]093	5 915	5 681	5 638	5 526	[illegible]887	4 5[illegible]	4 416	[illegible]
ν (kK)	49 162	47 712	45 82	45 [illegible]	[illegible]	39 411	36 [illegible]	35 62	[illegible]
f	[illegible]	0 07	0 244	0 024	0 1[illegible]	0 245	0 009	0 008	1 006
log ε*	2 376	3 467	4 0[illegible]	3 004	3 638	4 009	2 583	2 495	4 648
$\Psi_{i\to j}$	7 →10 6 →12	4 →9 5 →10	3 →11 5 →9	6 →9 7 →12 6 →10	3 →10	7 →9	6 →9 7 →12 6 →10	5 →9 3 →11 5 →10	8 →9

ε* = f · 41700

	9	8	7	6	5	4	3	2	1
ΔE (eV)	6 05	5 88	5 646	5 434	5 112	4 999	4 693	4 561	4 323
ν (kK)	48 797	44 426	45 536	43 329	41 258	40 318	37 042	36 787	34 787
f	0 357	0 238	0 215	0 021	0.157	0 241	0 092	0 348	0 402
log ε*	3 357	3 997	3.953	2 944	3.817	4.002	3 582	4.161	4 224
$\Psi_{i\to j}$	7 →11 6 →12	9 →12 6 →10	9 →13 8 →11	7 →10 9 →13	9 →11 8 →10 7 →10	7 →10 9 →10	6 →10 9 →12 8 →12	3 →10 9 →10 9 →11	9 →10

ε* = f · 41700

	10	9	8	6-7	5	4	3	2	1
ΔE (eV)	6 031	5 355	5 772	[illegible]	5 341	5 142	4 865	4 221	4 055
ν (kK)	48 641	47 228	46 554	[illegible]	43 078	41 477	39 238	34 048	32 706
f	0 113	0 567	0 112	[illegible]	0 069	0 274	0 136	0 220	0 446
log ε*	3 67	4 37	3 70	[illegible]	[illegible]	4 05	3.75	3 96	4 27
$\Psi_{i\to j}$	9 →13 3 →11	7 →11 6 →11 5 →10	9 →14 5 →10 6 →11	9 →12 7 →11 5 →10 3 →10 7 →10 9 →13	6 →10 7 →11 7 →10	8 →10 9 →12 7 →10	7 →10 6 →11 9 →11	9 →11	9 →10

ε* = f · 41700

	9	8	7	6	5	4	3	2	1
ΔE (eV)	6 15	5 739	5 577	5.453	5.325	4 956	4.315	4 229	3.282
ν (kK)	49600	46 292	44 981	43.98	42.948	39 974	34.799	34.143	26.467
f	0 054	0 091	0 035	0 056	0 375	0.26	0.003	0.304	0.864
log ε*	3 349	3 58	3.169	3 37	4 195	4 036	2 112	4 103	4 557
$\Psi_{i\to j}$	5 →10	8 →11 9 →14 7 →13	9 →13 5 →10 9 →12	9 →12 6 →11 9 →13	7 →10 9 →11	8 →13 9 →11 8 →10	6 →10 9 →12	3 →10 9 →11	9 →10

Fig. 1. Experimental and theoretical (PPP) UV data of flavonoids

explanations given on the basis of the resonance theory.

The results obtained by means of the CNDO/S method are indicated in Figures 2-6. The computation program, modified by us, permitted the graphic visualization of the theoretical spectra, thus providing a ready comparison with the experimental data.

The above Figures obviously demonstrate that CNDO/S, as an "all valence" method, reproduces the experimental data - both the places of the bands and the intensity relations - much better than the PPP procedure. It is also shown that the bands appearing as homogeneous in the experimental spectra are reproduced in form of more complex bands, indicating that the above virtually homogeneous bands are due to several simultaneous electron excitations.

In the case of chromone (Fig. 2) each transition is of $\pi \rightarrow \pi^*$ character. The $n \rightarrow \pi^*$ transition, being overlapped by the high intensity $\pi \rightarrow \pi^*$ transition, can not be identified.

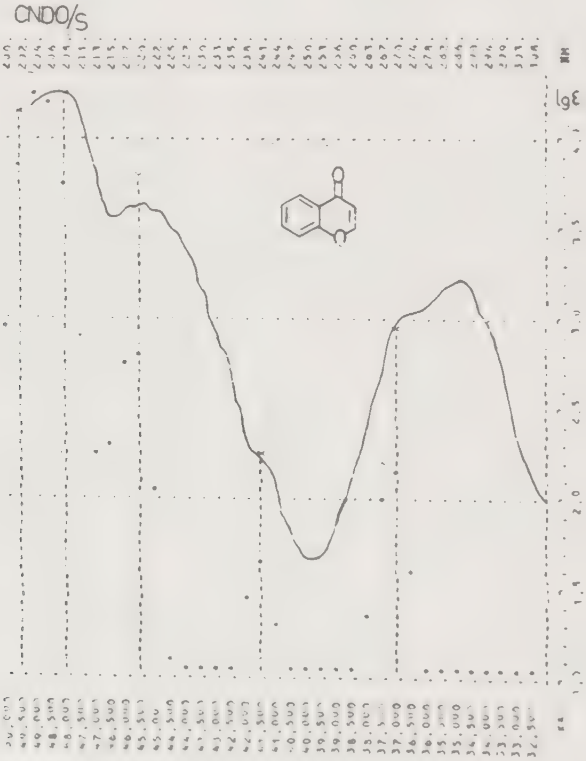
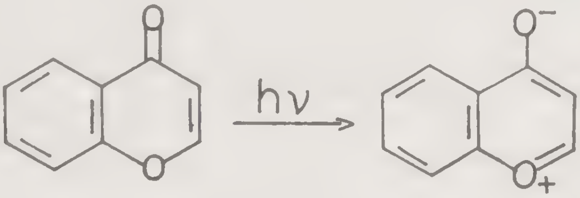
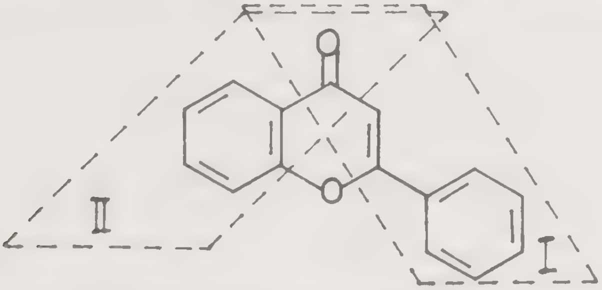


Fig. 2. Calculated /----/ and experimental /——/ UV spectra of chromone

The orbital coefficients of the wave functions obtained by means of the CNDO/S method showed the transition near 290 nm to be localized on the benzoyl group. The $\pi \rightarrow \pi^*$ transition near 220 nm corresponds to an E.T. (Electron Transfer) band, which can be formally described by the following resonance structures:

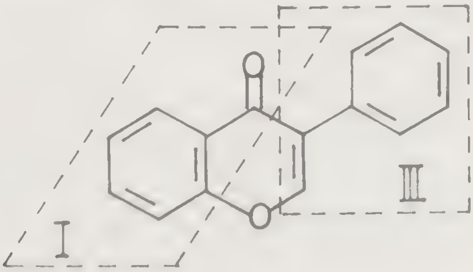


The transitions appearing in the spectrum of flavone (Fig. 3) are of $\pi \rightarrow \pi^*$ character assignable - on the basis of the orbital coefficients - to the following excitations; the bands near 280 and 250 nm are assigned, [17] respectively, to the cinnamoyl- (I) and benzoyl- (II) chromophores:



The bands in the spectrum of isoflavone (Fig. 4) near 300 and 240 nm are peculiar to $\pi \rightarrow \pi^*$ transitions; $n \longrightarrow \pi^*$ transition can be observed only in cyclohexane.

The comparison of the spectra of flavone and isoflavone shows similar intensities, but different places of the bands, indicating a conjugation of smaller extent in the molecule of isoflavone. The bands at higher and lower wavelenghts can be attributed to the styryl- (III) and benzoyl- (I) chromophore, respectively.



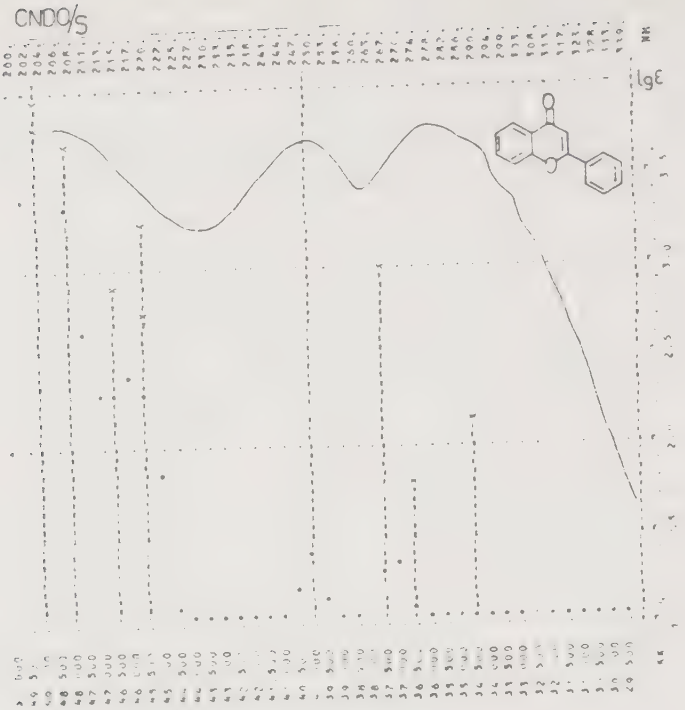


Fig. 3. Calculated /----/ and experimental /——/ UV spectra of flavone

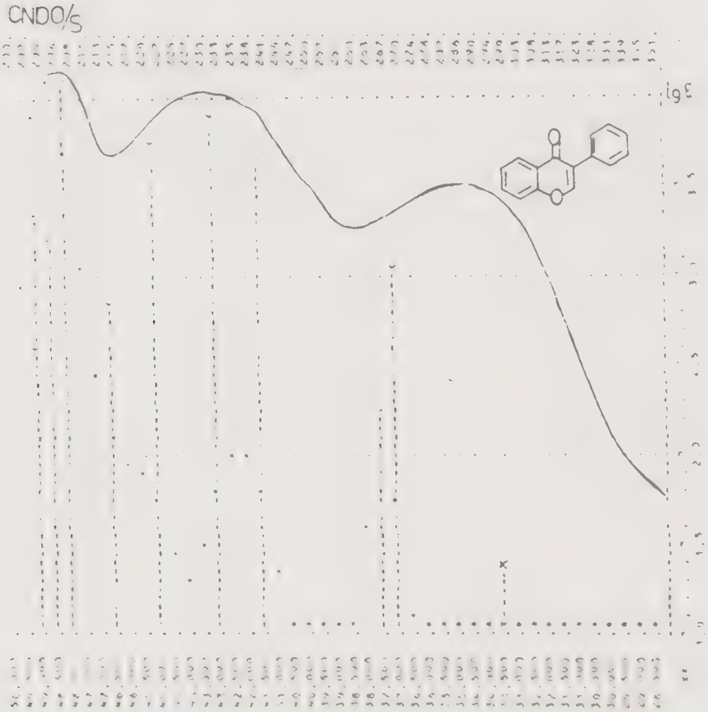


Fig. 4. Calculated /----/ and experimental /——/ UV spectra of isoflavone

The analysis of the wave functions obtained for aurone (Fig. 5) demonstrates that the intense absorption at 290 nm belongs to the benzoyl chromophore and the band near 400 nm is assigned to the cinnamoyl chromorphore. The band of highest wavelenght can be formally attributed to the absorption of flavone near 280 nm. This observed bathochromic shift may be mainly due to either the strained exocyclic C=C system or to the quasi-aromatic character of the pyrone ring.

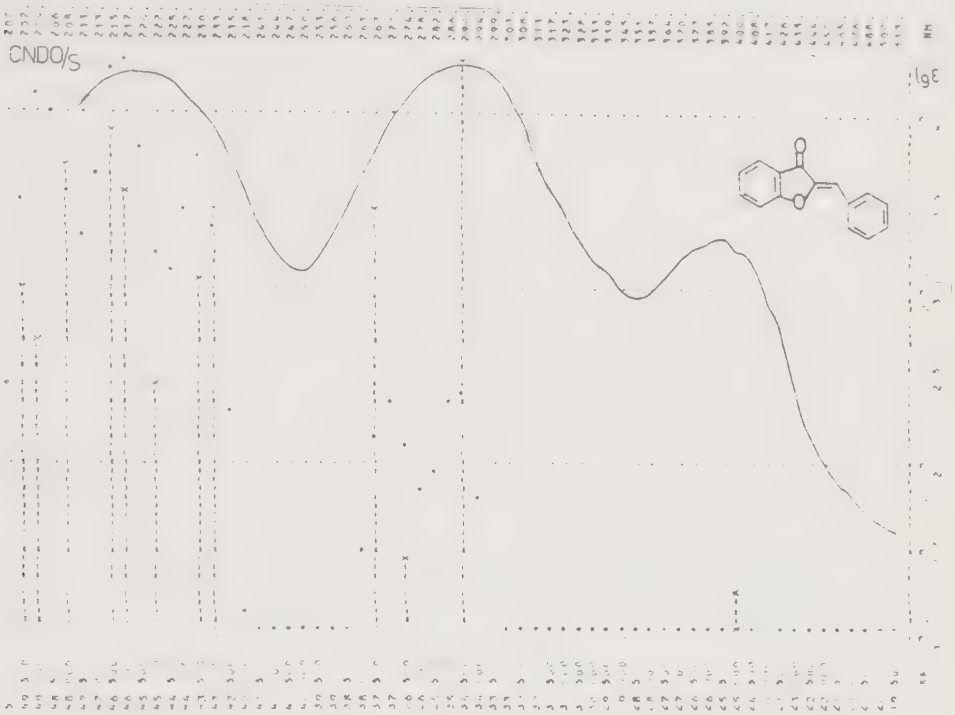


Fig. 5. Calculated /----/ and experimental /——/ UV spectra of aurone

In the case of chalcone (Fig. 6) the bands near 220 and 280 nm can be assigned to the benzoyl- and cinnamoyl chromophore, respectively.

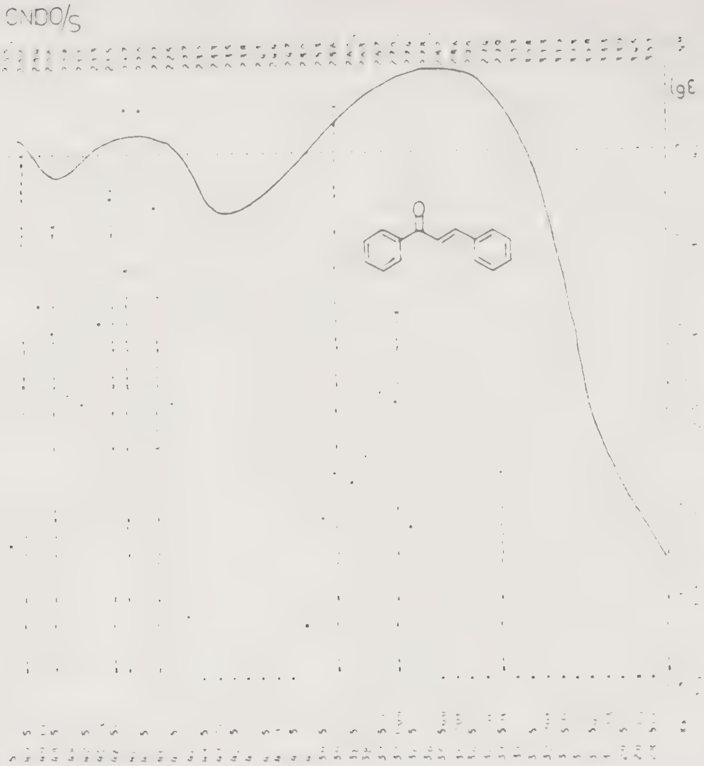


Fig. 6. Calculated /-----/ and experimental /——/ UV spectra of chalcone

Conclusion

The results of the CNDO/S calculations reproduce the experimental spectrum better than those of the PPP method. If the data of the quantum chemical calculations correspond well with the experimental system, other parameters (e.g. dipole moment, reactivity, electron density, ^{13}C-NMR chemical shift) of such calculations can be applied for the explanation of the additional chemical properties [18]. The agreement between the calculated data and the experimental values provides a good opportunity for controlling of the reliability of the quantum chemical calculation methods.

Based on the above results, quantum chemical studies are in progress for investigating the structural properties of substituted flavonoid derivatives, as well as those of the analogues carrying sulphur or nitrogen atom in the heterocyclic ring.

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Flavonoids and Bioflavonoids, 1985
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ETHYLENE CARBONATE, A REGIOSELECTIVE REACTANT FOR HYDROXYETHYLATION OF FLAVONOIDS

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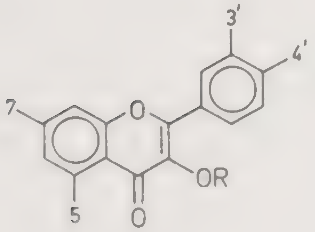
INTRODUCTION

For about two decades, O-ß-hydroxyethylrutosides have been used therapeutically for the treatment of symptoms of chronic venous insufficiency, the treatment of varicose veins, the treatment of haemorrhoidal conditions, the treatment of diabetic retinopathy and are used to prevent lesions induced by irradiation. The O-ß-hydroxyethyl derivatives of rutin are proved to possess beneficial influence on the capillary permeability and fragility and to have antiinflammatory properties (1). Due to the method of synthesis, the O-ß-hydroxyethyl rutosides are a standardized mixture of compounds with different degrees of hydroxyethylation (Figure 1).



%	3'	4'	5	7	R
50.1	—	—	OH	—	Rutinosyl
20.9	OH	—	OH	—	"
11.7	—	OH	OH	—	"
7.3	—	OH	OH	OH	"
<0.1	OH	OH	OH	—	"
5.1	—	—	—	—	"
0.5	OH	OH	OH	OH	"
3.4	—	—	OH	—	CH_2CH_2OH

—: OCH_2CH_2OH

Fig. 1:
Standardized mixture of O-ß-hydroxyethylrutosides

The components differ in their pharmacokinetic behaviour due to their different lipophilicities. This has been shown by Kuhnz et al. (2) by separating some of the O-ß-hydroethylrutosides by means of HPLC (Figure 2).

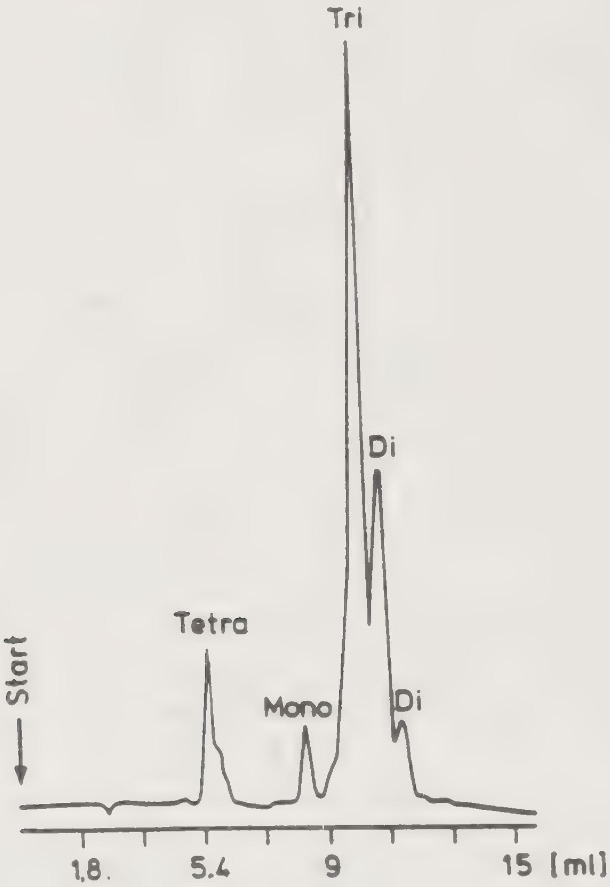


Fig. 2:
Separation of O-ß-hydroxyethylrutosides by means of reversed phase HPLC.

These differences in hydrophilic and lipophilic behaviour may influence pharmacokinetic parameters like absorption, distribution, metabolism and excretion (3). Protein binding experiments (Figure 3) and the ratio of urinary and biliary excretion in animal and clinical pharmacology studies support this hypothesis (Figure 4).

Protein binding of hydroxyethylated rutosides

7-Monohydroxyethylrutosides	52 %
3',7-Dihydroxyethylrutosides 4',7-Dihydroxyethylrutosides	21 %
3',4',7-Trihydroxyethylrutosides	11 %
3',4',5,7-Tetrahydroxyethylrutosides	5 %

Fig. 3:
Protein binding of O-ß-hydroxyethylrutosides in human serum in vitro.

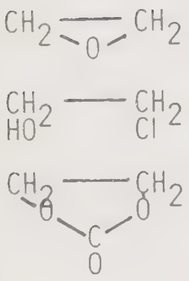
Excretion of hydroxyethylated rutosides in rats following i.v. application in percent of dose.

compound	time	urine	bladder	ratio
7-Monohydroxyethylrutoside	24	17	65	0,26
4',7,-Dihydroxyethylrutoside	24	13	64	0,20
3',4',7-Trihydroxyethyl rutoside	24	19	66	0,28
3',4',5,7-Tetrahydroxyethyl rutoside	24	54	29	1,86

Fig. 4:
Urinary and biliary excretion of O-ß-hydroxyethylrutosides following i.v. administration to rats.

METHODS

The synthetic methods available (Figure 5) for synthesizing the O-ß-hydroxyethylrutosides are the ethylene chlorohydrin and the ethylene oxide methods (4, 5).



	b.p.	MAK
ethylene oxide	10.7	50 cm^3/m^3
ethylene chlorohydrin	127.9	5 cm^3/m^3
ethylene carbonate	236	---

Fig. 5:
Available synthetic methods for hydroxyethylation of flavonoids.

The disadvantages of both methods - besides the toxicity of the reactants, ethylene oxide and ethylene chlorohydrin - are the limited reproducibility, the low yield, the stoichiometry of the reaction consuming a great excess of reactants, and the lack of regioselectivity.

Ethylene carbonate is a reactant of low toxicity. It is normally used as a solvent. The LD_{50} in rats is about 10 g/kg p.o. Inhalation of air saturated with ethylene carbonate did not provoke any case of death in rats. Skin irritability and eye irritation in rabbits is low. Ethylene carbonate is degradable and 1 g/l is not toxic to fishes (6).

For the reaction with ethylene carbonate quercetin was used as a model reactant for flavonoids, avoiding any interference with the glucosidic OH groups.

The scheme of the reaction between ethylene carbonate or propylene carbonate, respectively, (2) and quercetin (1) is shown in figure 6.

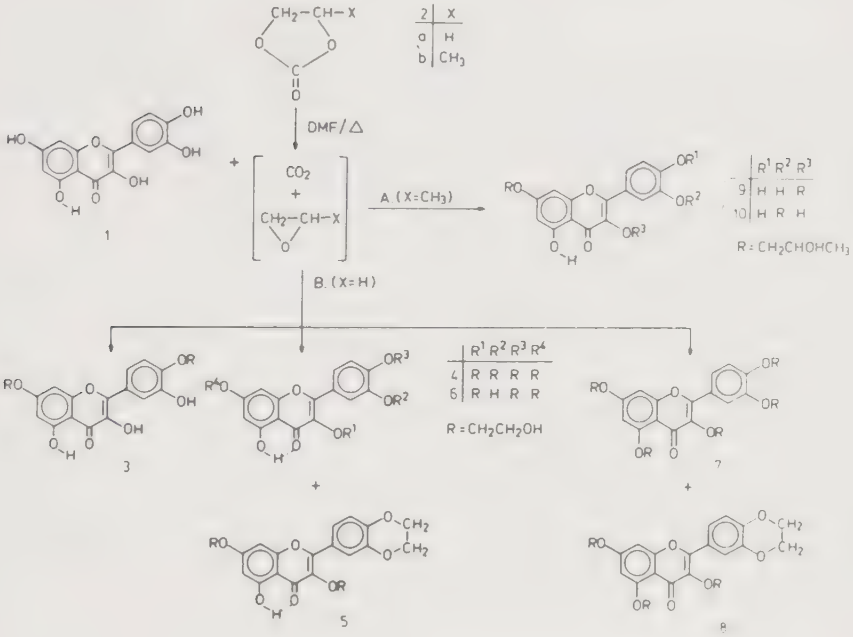


Fig. 6:
Scheme of the reaction between quercetin (1) and ethylene/propylene carbonate (2).

30 mmol of quercetin and 235 mmol ethylene carbonate, that means an 1.6 fold excess of the activated carbonate,was heated in dry dimethylformamide for 6 hours at 170 - 180° C resulting in 3,3',4',5,7-penta-(ß-hydroxyethoxy)-flavone in 55 - 60 % yield (7).

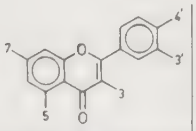
3',4'-Dioxyethylene-3,5,7-tri-(ß-hydroxyethoxy)-flavone (8) resulted from the filtrate of (7) in 3% yield. This dioxyethylene-bridged derivative is a new type of flavonoid with interesting partition behaviour.

Using an equimolar ratio of quercetin and ethylene carbonate related to the number of OH-functions, four hours heating in dimethylformamide gave 5-hydroxy-3,3',4',7-tetra(ß-hydroxyethoxy)-flavone (4) in 50 - 55 % yield. Under the same experimental conditions 3,3',5-trihydroxy-4',7-di-(ß-hydroxyethoxy)-flavone is obtained after 1 hour heating, in 15 - 20% yield (3).

3',4'-Dioxyethylene-3,7-di-(ß-hydroxyethoxy)-5-hydroxyflavone (5) was obtained from the filtrate of the reaction mixture leading to (4), in a yield of 5 - 10% by extraction with methylene chloride.

3',5-Dihydroxy-3,4',7-tri-(ß-hydroxyethoxy)-flavone (6) was obtained from the filtrate of (5) in a yield of 5 - 10%, too; (6) and (5) differ in their lipophilic/hydrophilic behaviour.

All compounds have been recrystallized until a stable melting point. The structures have been proved by means of NMR- and mass spectra. The chemical shifts of the aromatic hydroxylgroups are in the expected region by comparison with the chemical shifts of known hydroxyflavones as shown in figure 7.



^{1}H-NMR (d_6-DMSO): δ (ppm)

	3-OH	3'-OH	4'-OH	5-OH	7-OH
Flavon-3-ol	9,67				
3',4'-Dihydroxyflavone		9,45	9,93		
Chrysin				12,86	10,95
Galangin	9,71			12,40	10,90
Quercetin	9,39	9,39	9,66	12,52	10,85
O-Hydroxyethyl:5-Hydroxy	—	—	—	12,62	—
" :5- "	—	⌐‾‾¬		12,55	—
" :3,5- "	9,58	—	—	12,45	—
" :3',5 "	—	9,12	—	12,63	—
" :3,3',5- "	9,58	9,09	—	12,44	—

— : $-CH_2CH_2OH$
⌐‾‾¬ : $-CH_2CH_2-$

Fig. 7:
Chemical shifts of the hydroxyl functions in various flavones and O-ß-hydroxyethyl derivatives of quercetin .

The degree of the reaction leading to penta-hydroxyethyl derivates in which the 5 hydroxyl group is hydroxyethylated may be analyzed with IR spectroscopy. Derivatives with a free 5-hydroxyl function show a C=O band at 1650 cm^{-1}, whereas the hydroxyethylated compounds possess that C=O vibration at 1620 cm^{-1} (Figure 8).

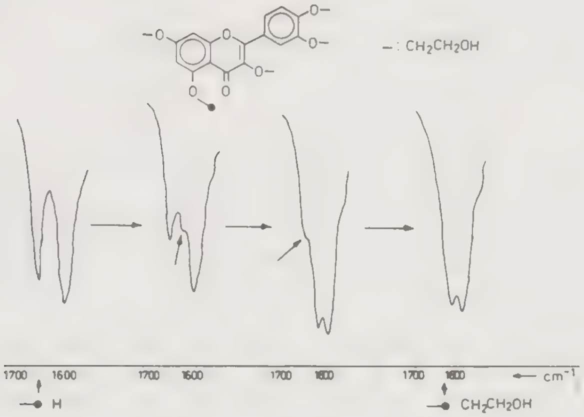


Fig. 8.:
IR spectroscopic determination of the free 5-hydroxyl function.

The attempts to synthesize 5-hydroxy-3,3',4',7-tetra(ß-hydroxypropoxy)-flavone and the related penta derivative using propylene carbonate instead of ethylene carbonate did not succeed. Only low amounts of (9) and (10) could be obtained in a yield of about 10 %. (9) could be proved to be 3',4',5-trihydroxy-3,7-di-(ß-hydroxypropoxy)-flavone, whereas (10) is 3,4',5-trihydroxy-3',7-di(ß-hydroxypropoxy)flavone.

Compared with the traditional methods for synthesizing O-ß-hydroxyethyl derivatives, the ethylene carbonate method leads to an improved regioselectivity. Though the mechanism of this reaction needs to be investigated in more detail, it is supposed that the improved regioselectivity is based on mainly two factors:

- the low reaction rate and
- the equimolar or nearly equimolar relation between the reactants.

The mechanism of the reaction is still uncertain. Cleavage of ethylene carbonate into CO_2 and ethylene oxide may happen under the thermal challenge and the moderate basicity of dimethylformamide, as known from the literature. But it is interesting that this reaction only succeeds in dimethylformamide as solvent. This may be due to a reactive intermediate between DMF and ethylene carbonate, too.

Some future possibilities with this regioselective method should be stressed. First of all it is to be investigated whether this method is usable more generally for rutin and other flavonoids, too.

The two types of molecules (Figure 9), both that with two O-ß-hydroxyethyl moieties at the 3' and 4' position and that bridged with one hydroxyethyl moiety, give the possibility for further derivatisation with the aim to find the compounds with optimal lipophilic behaviour. Alkylation of the free 5-OH-function leads to increased lipophilicity with sufficient water solubility. Derivatisation with dodecanoyl bromide leads to a product with tenside character.

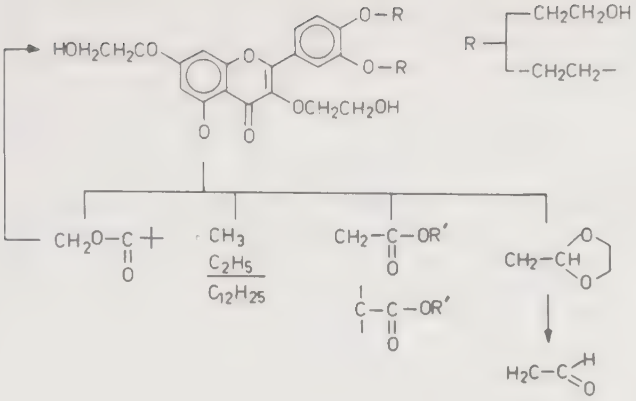


Fig. 9:
Derivatisation of 5-hydroxy-3,3',4',7-tetra-ß-hydroxyethoxyflavones and related flavones.

The pivaloyl derivative is expected to be an easily cleavable, more lipophilic pro-drug with improved rate and degree of absorption. Isobutyric acid derivatives contain the moiety said to be responsible for lipid lowering efficacy and the acetic acid derivatives have the moiety said to be responsible for inhibition of the aggregation of thrombocytes. Last but not least, the synthesis of derivatives suitable for the development of radio-or enzyme immuno-assays is possible via the aldehyde function.

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POLYPHENOL COMPLEXATION

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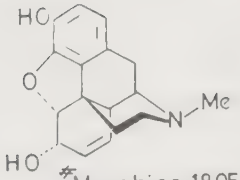
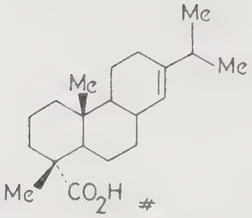
INTRODUCTION

Natural products lie at the heart of organic chemistry and studies of substances present in living matter continue to enrich its development. Liebig's *Handbuch der OrganischenChemie* of 1843 listed some 2000 substances of natural origin and since that date many thousands more have been described. With the emergence in the 20th century of biochemistry there came the realisation that for many natural products (Figure 1*) - the ubiquitous intermediates of primary metabolism - a distinctive role in the life of most if not all organisms could be assigned. The pathways by which they are synthesised are similar if not identical in all organisms and because of their primacy in living systems they are frequently referred to as primary or intermediary metabolites. In contrast an infinitely greater body of natural substances (Figure $1^{\#}$) - alkaloids, mycotoxins, phenols, polyenes, polyacetylenes, terpenes, etc. - occur sporadically throughout Nature. Moreover, they appear to play no explicit role in the welfare and economy of the producing organism. Substances in this category, because of their apparently subsidiary role, are commonly referred to as secondary metabolites /1/2/. Whilst this distinction between primary and secondary products of metabolism is useful in a didactic way, it should always be borne in mind that the boundary which separates them is often diffuse and ambiguous. Nevertheless, the question of the function (if any) of these 'children of nature' is one of fundamental biological importance and significance. Various propositions have been advanced /1/2/3/, but fiction still tends to dominate fact in this area of scientific enquiry.

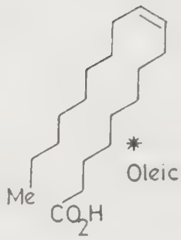
The vastly expanded and increasingly complex array of physical methods which the organic chemist now has at his command to solve structural problems



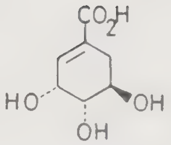
#Morphine,1805



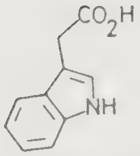
#Abietic acid ,1826



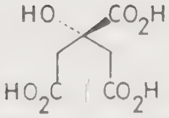
*Oleic acid , 1846



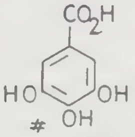
*Shikimic acid , 1885



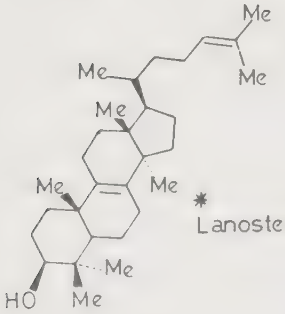
*Indole acetic acid , 1880



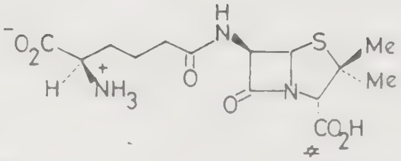
*Citric acid,1784



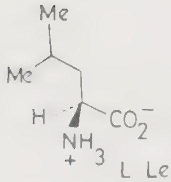
#Gallic acid,~1770



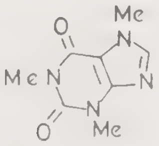
*Lanosterol ,1872



*Penicillin N , 1954



*L Leucine ,1818



#Caffeine ,1820

Figure 1. Natural Products - Primary and Secondary Metabolites

has over the past 25 years transformed natural products chemistry. Relieved of much of the burden and tedium of structure proof and determination, this has brought a reappraisal of many of its objectives. Of itself structure determination no longer remains the principal goal. Increasingly attention focusses on the way in which 'natural products' participate in the dynamics of living system. Nowhere has this mushrooming interest become more evident than with plant polyphenols. Polyphenols (*syn*-vegetable tannins-proanthocyanidins and esters of gallic acid, Figure 2) constitute a distinctive group of higher plant secondary metabolites. Their uniqueness lies not only in their polyphenolic character but also in their propensity to complex strongly with proteins, carbohydrates, nucleic acids and alkaloids. These distinctive properties have influenced several workers to examine the possible role of polyphenols in higher plant secondary metabolism. Likewise these lie at the basis of a number of phenomena of considerable practical significance. The characteristics of many plant products, such as their taste and astringency, palatability, nutritional value, pharmacological and toxic effects, and their microbial decomposition in the formation of soils are all substantially influenced by the polyphenols they contain. There is currently a renewed interest in the nature of 'non-bonded' interactions which occur between atoms and molecules and, in particular, a desire to have a fuller appreciation of the role such interactions play in biological systems. It is thus timely that thoroughgoing investigations of the interactions - both 'non-bonded' and those which result in the formation of chemical bonds - between plant polyphenols and proteins, polysaccharides and alkaloids should have now begun. Such investigations are important not only because of fundamental intrinsic scientific interests, but also because of the potential practical benefits which may ultimately emerge.

Studies of the reversible association of polyphenols with proteins have a long history - one of the first scientific papers on this subject is that of Sir Humphry Davy in 1803. Early work such as this demonstrated some of the macroscopic features of the complexation and enabled several empirical definitions of the term vegetable tannin to be advanced /4/5/6/. Such studies were nevertheless dogged by the inability to define the homogeneity and molecular composition of the plant polyphenols which were employed. It is the recent advances in this latter area /6/7/ which now make such studies feasible at last.

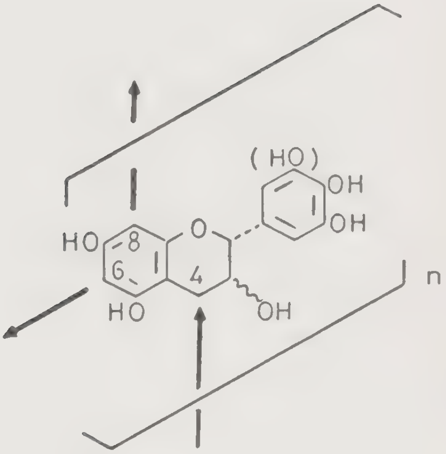
PLANT POLYPHENOLS - CLASSIFICATION AND STRUCTURE

The word tannin has been used in a very wide sense in the botanical literature although the reproach that everything which gives a blue colour with ferric salts has been called a tannin is probably not entirely justified. A firm definition of what constitutes a vegetable tannin is not easy to give but the original implications of the word clearly indicated a plant material which produces leather from hide. Bate-Smith and Swain /4/ have adopted the earlier ideas of White /5/ in formulating a definition of vegetable tannins which they classified as 'water-soluble phenolic compounds having molecular weights between 500 and 3000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins.' Some still prefer the description 'tannin' which they find useful in discussion precisely because of its lack of definition. Terminologically and scientifically plant polyphenol is to be preferred.

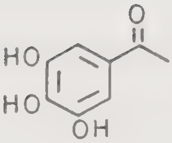
Plant polyphenols are broadly divisible into two major groups - the proanthocyanidins and polyesters based on gallic acid and/or hexahydroxydiphenic acid and their derivatives, Figure 2. In the earlier literature the former category corresponds to that of the condensed tannins and the latter to the hydrolysable tannins (Haslam /6/7/8/). As markers of evolutionary trends in plants proanthocyanidins are present in the most primitive of vascular plants such as ferns and gymnosperms, whereas esters of gallic and hexahydroxydiphenic acids appear for the first time in dicotyledons. Plant polyphenols possess a number of molecular characteristics which embody their unique and distinctive properties. Not only do they carry a multiplicity of phenolic groups but also they are found with a range of molecular weights (proanthocyanidins up to 20,000 and esters of gallic acid and its derivatives up to 3000) typically intermediate between those of macromolecular species such as proteins and polysaccharides and other primary and secondary metabolites (generally up to 500).

Plant proanthocyanidins are based structurally on a polyflavan-3-ol structure (Figure 2) and from the phylogenetic point of view first appeared as plants developed a vascular character - in many ferns for example where proanthocyanidins are invariably found in polymeric forms. More generally, in plant tissues a spectrum of products may be discerned /9/10/ ranging from the monomers -(+)-catechin and (-)-epicatechin - through the various dimers and simple oligomers, all of which are freely soluble, to those

Proanthocyanidins
(M.Wt. → 20,000?)
Associated with
"Woodiness"



Galloyl-esters
(M.Wt. → 1250)



Hexahydroxydiphenoyl-esters
(M.Wt. → 2800)

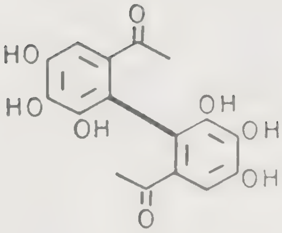


Figure 2. Polyphenols - Classification

polymeric forms which are insoluble in water and the usual range of organic solvents. The latter group are indeed generally characteristic of all proanthocyanidin containing tissues where they invariably predominate over the more soluble forms - typically by as much as 10 or 20:1. Present evidence (*vide infra*) strongly suggests that these proanthocyanidins are probably covalently bound to a polysaccharide matrix with the plant cell (Figure 3).

Recent work /8/11/12/ has demonstrated the widespread occurrence in plants of esters of gallic acid and the biosynthetically related hexahydroxydiphenic acid with D-glucose, Figure 2. In direct contrast to other natural phenolic esters, such as those of the hydroxycinnamic acids (ubiquitous) or those of p-hydroxybenzoic and salicylic acids (rare), which invariably occur as mono- and occasionally bis-esters with polyols, gallic acid and hexahydroxydiphenic acid and their derivatives are found in a range of esterified forms usually with D-glucose and with molecular weights spanning the range 500 to at least 2800. These substances (gallotannins and ellagitannins) constitute the second major class of plant polyphenols and occur solely in dicotyledons.

A number of important properties derive from the combination of moderate molecular size and polyphenolic character in plant polyphenols; chief amongst these is their ability to reversibly complex with various natural macromolecules and alkaloids. Historically the umbrella nomenclature - vegetable tannin - derives from these properties. It is tempting to suggest on this basis similar roles in plant metabolism for both proanthocyanidins and the polyphenolic esters. Comparative studies nevertheless suggest some tentative, potentially fascinating relationships. The two forms of metabolism are not mutually exclusive, but *often* where tissues are rich in proanthocyanidins, synthesis of gallic acid and its derivatives is minimal. Similarly, an analysis of different tissues (fruit, leaves, stem, root) of the same plant frequently reveals differing patterns of polyphenol distribution. Finally, in some plants such as *Camellia sinensis*, the leaves are rich in flavan-3-ols - principally as their 3-O-gallate esters - but the levels of proanthocyanidin synthesis are minimal.

POLYPHENOL-PROTEIN REVERSIBLE COMPLEXATION

Polyphenol reversible association with proteins may be studied in solution or by an examination of the precipitation which ensues after complexation. Various physicochemical techniques are appropriate to such analyses (Figure 4). They lead correspondingly to information of a thermo-

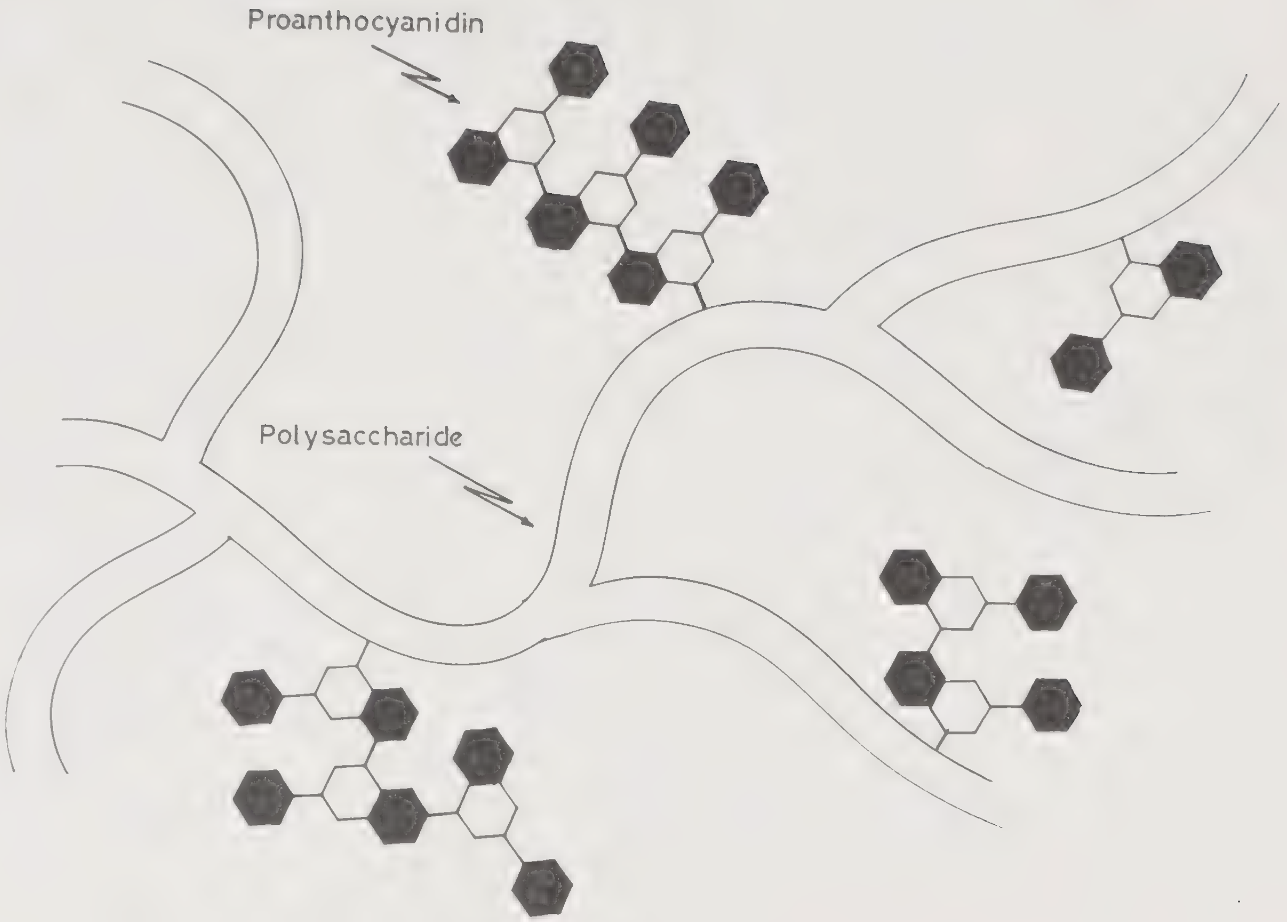


Figure 3. Insoluble Polymeric Proanthocyanidins - A Pictorial View

dynamic nature (eg. $\Delta G^{\theta,tr}$ - the free energy of transfer of a polyphenol molecule from water to a protein solution) or to stoichiometric data

$$\begin{array}{ll} & \text{Protein} \; + \; \text{Polyphenol} \\ \text{SOLUTION} & \qquad \uparrow\downarrow \\ & [\text{Protein} \; . \; \text{Polyphenol}_n] \\ \text{PRECIPITATION} & \qquad \uparrow\downarrow \\ & [\text{Protein} \; . \; \text{Polyphenol}_m] \end{array}$$

Solution
(a) ^1H and ^{13}C NMR
(b) Microcalorimetry ($\Delta H^{\theta,tr}$)
(c) Equilibrium dialysis ($\Delta G^{\theta,tr}$)

Precipitation
(a) Turbidimetric titration
(b) Radiolabelled protein, L.G. Butler /13/
(c) Haemanalysis, E.C. Bate-Smith /14/

Figure 4. Analysis of Polyphenol-Protein Association

concerning the composition of protein - polyphenol precipitates. There is a broad comparability in the information obtained from solution or precipitation experiments. They also clearly demonstrate that whilst a given polyphenol may exhibit substantially different affinities for a series of different proteins (Hagerman and Butler, /13/15/), for a given group of polyphenols their comparative behaviour with one protein generally parallels the behaviour with other quite different proteins. The reversible association of polyphenols with protein is an extension of the binding of simple phenols and is to some degree a function of the number and orientation of phenolic groups in the substrate. At or near physiological pH, amongst simple phenols for example, the binding of resorcinol is much weaker than that of catechol, pyrogallol or methyl gallate. At more acidic pH's the overall strength of association is reduced and hydrophobic, as opposed to hydrogen bonding, interactions probably assume a greater significance. The association on the basis of present information appears to be a relatively non-specific surface phenomenon. The effectiveness of plant polyphenols as complexing agents for proteins derives from the fact that they are polydentate ligands with a multiplicity of potential binding sites provided by the numerous phenolic groups and aryl rings on the periphery of the molecule. Cooperativity of complexation to the protein surface is thus observed with polyphenols which may also, because of their molecular size and structure, form stable cross-linked structures with different protein molecules.

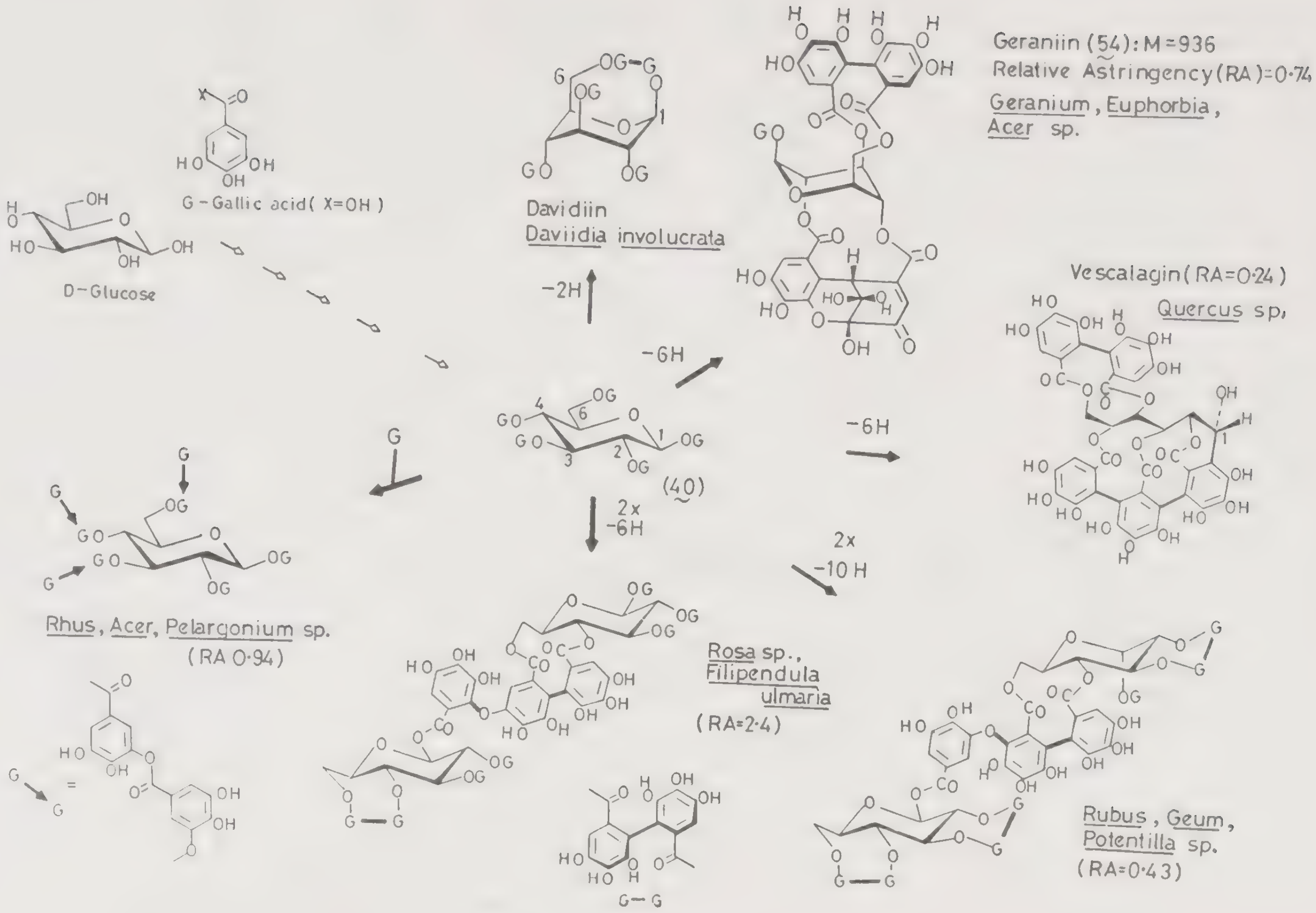


Figure 5. Polyphenol Complexation to Protein

Recent studies of gallic acid metabolism in higher plants /8/18/19/20/ has opened the way for a biosynthetically related study of the structure - activity relationships in the association of polyphenols with proteins. Products of gallic acid metabolism (Figure 5) in plants provides a series of polyphenols of defined molecular shape and size /18/19/20/. Quantitative measurements of the bonding of various phenols and polyphenols to the protein bovine serum albumin (BSA) were obtained by equilibrium dialysis and microcalorimetry /16/17/. These results now permit some of the facets of polyphenol structure necessary for enhanced complexation with proteins in solution to be delineated for the first time; the analysis is based principally on $\Delta G^{\theta,tr}$ values:

(i) *Complexation is dependent on protein type and pH.* Association is essentially a surface phenomenon, maximised at or near the isoelectric point of the protein and occurs principally via the intermediacy of hydrogen bonds and hydrophobic interactions.

(ii) *Molecular size of the polyphenol.* In, for example, the galloyl-D-glucose series the efficacy of binding increases exponentially in the series tri → tetra → penta (M_R-636, 788 and 940).

(iii) *Conformational flexibility of the polyphenol.* When conformational restraints are placed on the polyphenolic substrate, then its capacity to complex, whatever its molecular size, is dramatically reduced (eg. *Rubus* dimer, polymeric proanthocyanidins). Complimentarity leading to enhanced complexation between the polydentate ligand (polyphenol) and the receptor (protein) is maximised by conformational mobility in both components (Figure 5: relative astringency is extent of binding compared to that of β-penta-O-galloyl-D-glucose).

Collectively these results fully complement those of Hagerman and Butler /13/15/ who showed that proline-rich and conformationally mobile proteins have high affinities for polyphenols and that, on occasion, such proteins may be preferentially precipitated in presence of other proteins. Thus complimentarity between the polydentate ligand (polyphenol) and the receptor (protein) is maximised by conformational flexibility in both components.

When polyphenols cause precipitation of proteins from solution, two situations may be envisaged. At low protein concentrations the polyphenol associates at one or more sites on the protein surface to give a mono-layer which is less hydrophilic than the protein itself (Figure 6a). Aggregation and precipitation then ensue. Where the protein concentration is high, the

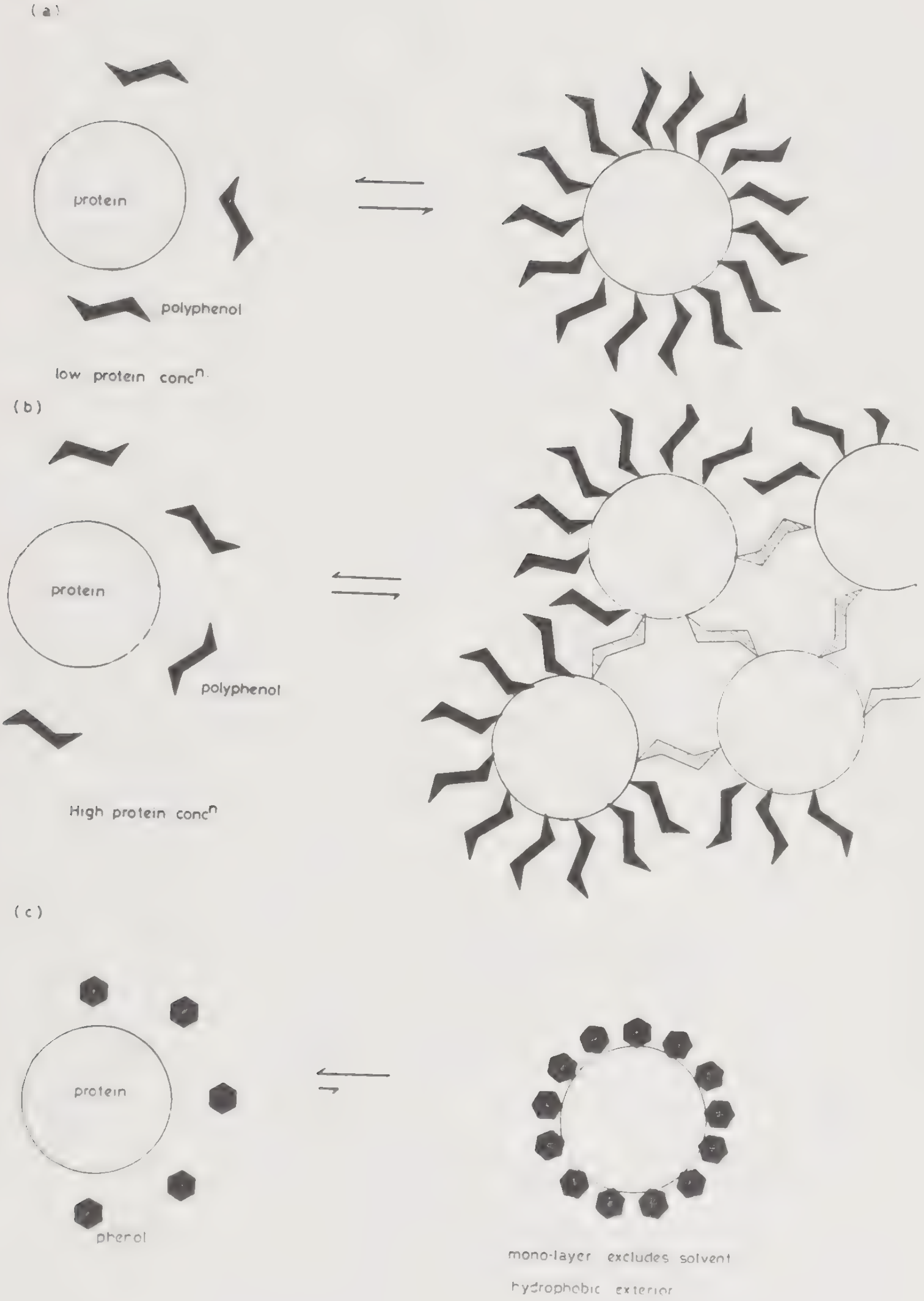


Figure 6. Polyphenol-Protein Precipitation

relatively hydrophobic surface layer is formed by complexation of the polyphenol on to the protein and by cross-linking of different protein molecules by the multi-dentate polyphenols (Figure 6b). Precipitation then follows as above. This tendency to cross-link protein molecules at higher protein concentrations explains the changing stoichiometry of the aggregates with changing protein concentrations - an observation first hinted at by Sir Humphry Davy. More polyphenol is thus required to precipitate proteins from dilute solution than from concentrated solutions. An interesting corollary of this hypothesis is that simple phenols such as pyrogallol and resorcinol should also be capable of precipitating proteins from solution if they can be maintained in solution at concentrations sufficient to push the equilibrium in favour of the protein - phenol complex and thus form a hydrophobic layer of simple phenol molecules on the protein surface (Figure 6c). For many simple phenols the limit is provided by their solubility in water, but it can be achieved with bovine serum albumin (3×10^{-5} molal) and pyrogallol (1 molal) and resorcinol (2 molal). Clearly, such factors are important when assessing the total astringency of some plant extracts rich in comparatively simple phenolics.

Circumstantial evidence also suggests that the complexation of polyphenols with proteins is modified by carbohydrates, other phenols and nitrogen containing metabolites. Correspondingly, the influence of polyphenols on the properties of plant materials - for example the changes in astringency of fruits as they ripen, or in storage - is substantially modified. Polyethylene oxides and amyloses ($M_R > 4000$) readily complex with polyphenols but to date quantitative studies have been severely limited by the availability of water-soluble polysaccharides with clearly defined molecular weights and molecular characteristics. Semi-quantitative studies show that the association of polyphenols with polysaccharides is - *in contrast to that with proteins* - broadly independent of pH. Molecular size and flexibility are likewise critical factors, but significantly where the polysaccharide can sequester the hydrophobic aryl residues of the polyphenol - holes in a crystal lattice (cellulose) or hydrophobic cavities (amylose and polysaccharide gels), then complexation is substantially enhanced (Figure 7). These changes in patterns of behaviour also imply important differences when the complexation of protein for polyphenols becomes competitive with polysaccharide.

Recent studies (Van Buren and Robinson /21/, Loomis /22/, Haslam /23/, and Hoff et al. /24/) have confirmed the principal means - hydrogen bonding

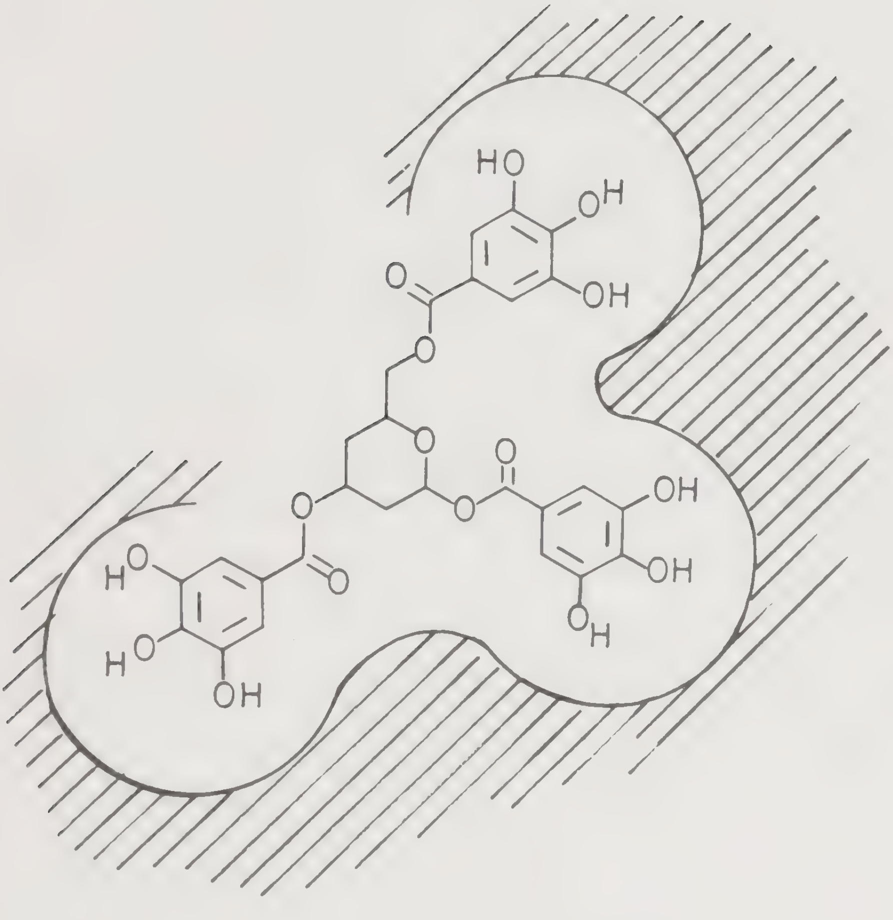


Figure 7. Cavity Sequestration of Polyphenols by Polysaccharides

and hydrophobic interactions - whereby polyphenols may associate with proteins. The relative importance which these two forms of non-covalent bonding play in the complexation is still uncertain. Whilst the traditional tendency has been to emphasise the part played by intramolecular hydrogen bonding between phenolic groups and the peptide bond, Hoff has recently hinted that hydrophobic effects may dominate the interaction. Evidence for the part played by both of these non-covalent interactions in phenol complexation comes from other sources (Falshaw, Bailey, Martin, Lilley, Magnolato and Haslam, unpublished observations). Thus X-ray and N.M.R. analysis show that caffeine complexes to phenolic substrates, such as chlorogenic acid, by a combination of phenolic hydrogen bonding to the cyclic keto-amide and a hydrophobic interaction (van der Waals attractions and exclusion of the solvent water) between the planar aryl and purine ring systems (Figure 8).

One of the key factors in this whole problem is, of course, the solvent water. To date it has been tacitly ignored. Biologists have been intrigued for a long time by the idea of structured water solvation shells around biological molecules and whether there are preferred binding sites for water on the substrate. It is natural therefore to ask what is the effect of bound water upon the properties of the biological substrate? Similarly what is the effect of the solvation shell on binding to the biological molecule by other species. Very little is known experimentally about this fundamental subject which with various ions constitutes the constant environment of biomolecules.

PLANT-HERBIVORE INTERACTIONS

It is a precept of modern chemical ecology that energy is unlikely to be 'wasted' in the production of secondary metabolites unless there is some compensating adaptive advantage to the organisms in question /25/. The biosynthesis of polyphenols involves a substantial input of energy and it might be expected, in the light of such assumptions, that there was a considerable compensating gain to polyphenol-containing plants. Equally, if these metabolites are the end-products of energy demanding syntheses, then it is reasonable to assume that there has been positive selection for their production /26/. In this context it is appropriate to comment very briefly on the results (Figure 5) in relation to theories of plant-herbivore interactions in which the repellant properties of plant polyphenols figure largely.

The results described show that there is a very wide variability in the protein-complexing capabilities of the polyphenolic biosynthetic 'end-products'

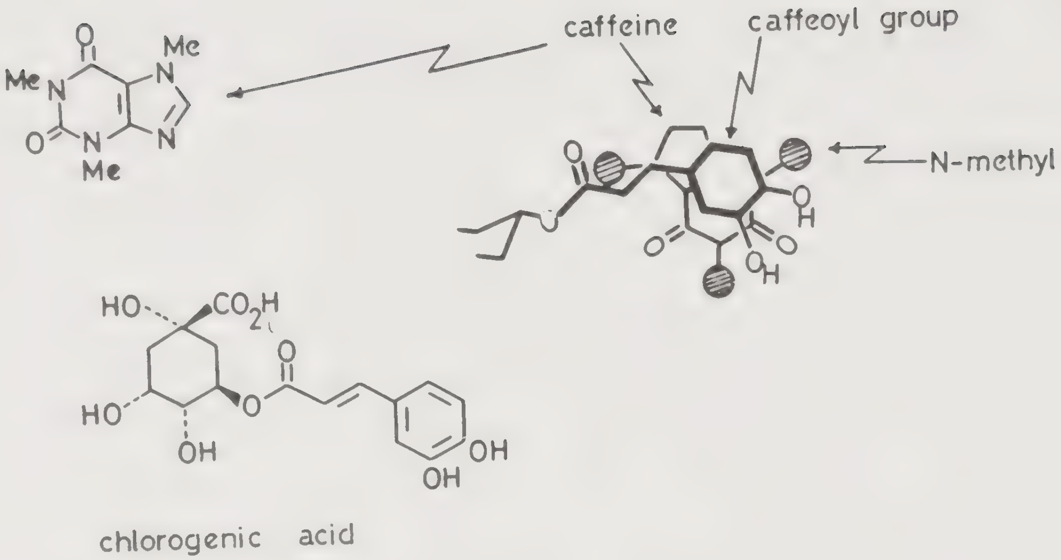


Figure 8. Potassium Caffeine Chlorogenate - X Ray Analysis

(Figure 5). There is no clear consistent pattern, nor is any correlation discernible between the apparent metabolic cost to the plant of its synthesis of a particular polyphenol and the subsequent capacity of that polyphenol to complex with protein and hence that polyphenol's astringency. If, as Rhoades has suggested /26/, there has been positive selection for the production of such metabolites, then this does not appear to be based solely, if at all, on the capacity of these polyphenols to bind protein. In many cases (Figure 5) the plant's subsequent chemical manipulation of the key pivotal biosynthetic intermediate -β-penta-O-galloyl-D-glucose is quite clearly counterproductive *vis a vis* the synthesis of metabolites with enhanced astringency. The evidence presently available suggests that although the retention of polyphenolic synthesis such as this in certain taxa may confer an advantage, a secondary benefit, on the plant and may well be the basis of selective pressures, it does not appear to support the proposition that the purpose of polyphenol metabolism is to specifically generate agents for the plant's defence. Protection, if it exists, would on the basis of this reasoning be a consequence of polyphenol formation, not a cause /3/.

POLYPHENOL-PROTEIN IRREVERSIBLE ASSOCIATION

The interaction of polyphenols and cell constituents such as proteins and polysaccharides may be a reversible or an irreversible process. Once a polyphenol has formed a complex with a protein (say) by the deployment of various non-covalent forces, covalent bonds, permanently linking the two species, may subsequently be forged in secondary chemical reactions and the association becomes an irreversible one. Under oxidative conditions, at or near physiological pH and with or without enzymic catalysis, phenols are readily transformed to quinones which may then react with nucleophilic groups (eg. $-SH$, $-NH_2$) on the protein (Figure 9). Particular examples of this phenomenon in Nature are the formation of the sclerotins (responsible for the exo-skeleton of insects /27/) and the tanned silks of saturnid moths /28/. In both of these examples considerable physical and chemical changes in the properties of the proteins ensue after reaction with simple phenolic oxidation products. When an insect cuticle is freshly secreted, it is flexible and pale; in a matter of hours it hardens and generally darkens. *o*-Dihydroxyphenols occasionally free or as glyosides secreted with the presclerotin, are enzymically hydrolysed to liberate the aglycone and oxidised to *o*-quinones, which react with nucleophilic groups on the protein to introduce exogenous cross-links between the polypeptide chains (Figure 9). Tanning of fibroin and

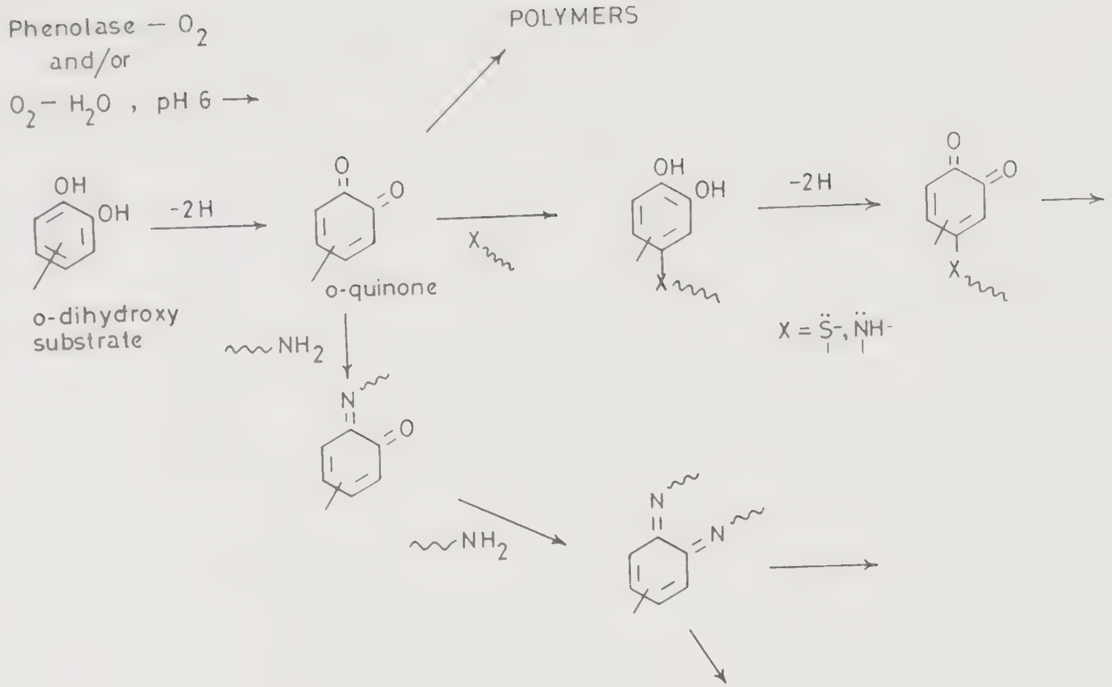


Figure 9. Protein-Polyphenol Interactions: Covalent Bond Formation

sericin in saturnid silks occurs by similar means. *o*-Quinones and related species formed during the oxidation of plant phenols by the ubiquitous polyphenoloxidases can similarly modify both cell constituents and plant pathogens, such as viruses.

The importance of polyphenols in brewing and the preparation of beverages has been recognised for a long time. When uninfected bottled beers are stored for any length of time they 'throw' a haze. Before a beer shows such a permanent haze at ambient temperatures, it may form a chill haze if cooled. Such hazes redissolve on warming and are presumably polyphenol-protein complexes formed reversibly. The major components of hazes are proteins, polypeptides, polyphenols, carbohydrates and traces of inorganic matter. Various theories have been put forward to explain the formation of permanent hazes and a general although vague consensus supports the view that they are produced by the gradual polymerisation of simple phenols, during storage, and the subsequent reaction of these polymers with proteins to give insoluble complexes /29/30/. Much of the published work on haze formation has implicated a group of polyphenols known generically as anthocyanogens, a useful but chemically imprecise term, which has led to some ambiguities in interpretation. Proanthocyanidins are the only anthocyanogens which have been shown unequivocally to occur in barley, malt, hops and beers; chief amongst these has been procyanidin B-3 (catechin 4α → 8-catechin) previously isolated from beer /16/31/32/.

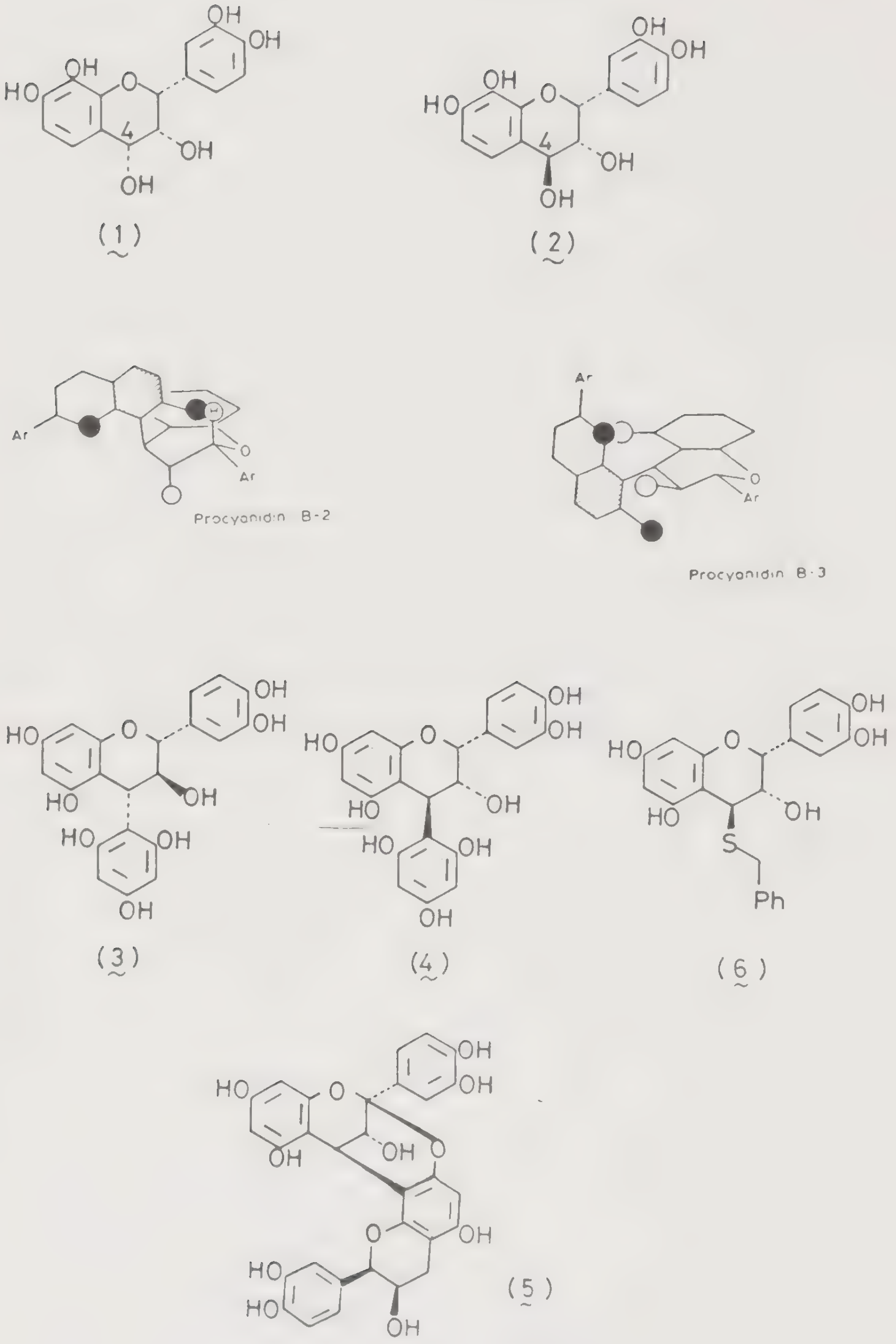
Hazes may be isolated, usually as black amorphous powders, from beers and other beverages by centrifugation. Decomposed with strong acid (5N HCl) they give a characteristic fingerprint of protein amino-acids typical of the presence of proteinaceous material. During the initial stages of this degradation the hydrolysate assumes a deep red colour ($\lambda_{max} \sim 535$ nm) characteristic of the pigment cyanidin.

It was considered at the outset that haze formation probably resulted, in some way, from the familiar acid-catalysed degradation of procyanidins present in beer, since this beverage has a pH typically around pH 4.0. The hydrolytic decomposition of various procyanidins - B-2 (epicatechin-4β → 8-epicatechin); B-3; B-4 (catechin-4α → 8-catechin) and B-5 (epicatechin-4β → 6-epicatechin) was therefore studied in acetonitrile - water - phosphoric acid mixtures, generally at 40°C, and over a range of pH values. Kinetics were monitored by HPLC analysis. The acid-catalysed solvolysis of the interflavan bond of procyanidin dimers is believed to proceed *via* the formation of

Table 1. First-order Rate Constants for the Acid-Catalysed Hydrolysis of Procyanidins in Acetonitrile-Water-Phosphoric Acid.

Compound	Temp. °C	pH*	$10^3 k/hr^{-1}$
Procyanidin B-2; Epicatechin-(4β-8) - Epicatechin	40	0.55	75
Procyanidin B-3; Catechin-(4α-8) - Catechin	40	0.55	71
Procyanidin B-4; Catechin-(4α-8) - Epicatechin	40	0.55	75
Procyanidin B-5; Epicatechin-(4β-6) - Epicatechin	40	0.55	30
(2*R*, 3*R*, 4*R*)-4-(2,4,6-Trihydroxy-phenyl)-flavan-3,3',4',5,7-pentaol (**3**)	40	0.55	65
(2*R*, 3*S*, 4*S*)-4-(2,4,6-Trihydroxyphenyl)-flavan-3,3',4',5,7-pentaol (**4**)	40	0.55	58
(2*R*, 3*S*, 4*S*)-4-Benzylthioflavan-3,3',4',5,7-pentaol (**6**)	40	0.55	18
Procyanidin B-2	25	0.55	10
Procyanidin B-2	30	0.55	22
Procyanidin B-2	35	0.55	51
Procyanidin B-2	45	0.55	169
Procyanidin B-2	25	0.95	5.2
Procyanidin B-2	35	0.95	17
Procyanidin B-2	25	1.49	1.7
Procyanidin B-2	35	1.49	7.5
Procyanidin B-2	25	0.25	63

* All measurements of pH relate to Acetonitrile-Water mixtures



Scheme

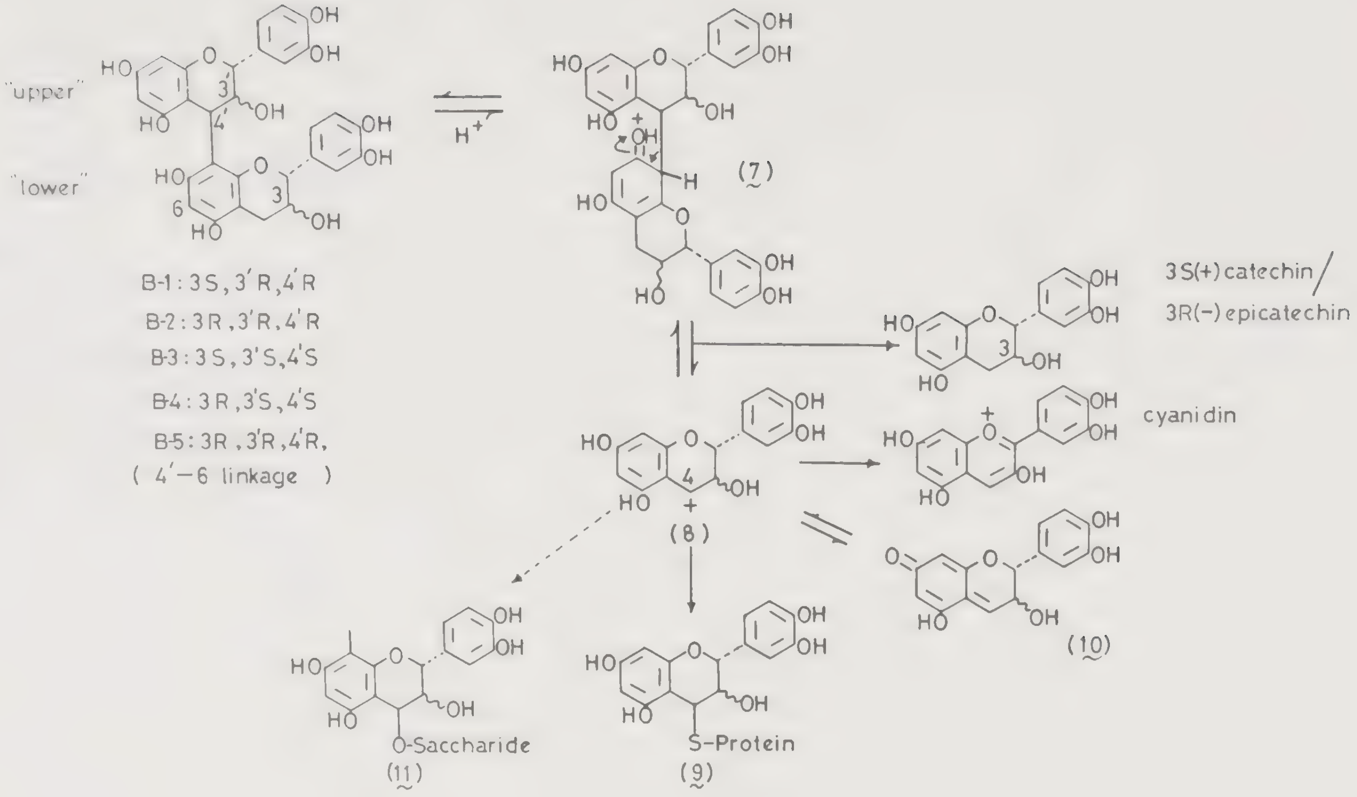


Figure 10. Procyanidin Acid-Catalysed Decomposition

the resonance-stabilised benzylic carbocation at C-4 in the 'upper' flavan-3-ol unit and of the appropriate flavan-3-ol - (-) - epicatechin or (+) - catechin - from the 'lower' - flavan-3-ol unit, Figure 10. Earlier work by Clark-Lewis and Mortimer /33/ with the diastereoisomeric flavan-3,4-diols, melacacidin (1) and isomelacacidin (2), had indicated that acid-catalysed solvolysis was facilitated in the isomer (2) where the hydroxyl group at C-4 occupies a quasi-axial position on the heterocyclic ring. This, it was argued, was due to the enhanced π-resonance stabilisation of the incipient C-4 carbocation, which is only possible when the C-4 hydroxyl group assumes a quasi-axial orientation. It was anticipated on the basis of this stereochemical analysis that B-2 and B-5 in which the 'lower' flavan-3-ol unit occupies a quasi-axial position at C-4 on the 'upper' flavan-3-ol unit, would undergo more facile acid-catalysed decomposition than the procyanidins B-3 and B-4 where the 'lower' flavan-3-ol unit of the dimer occupies a quasi-equatorial position. In the event this anticipation was not realised in practice. The initial rates of hydrolysis of procyanidins B-1, B-2, B-3 and B-4 were (Table 1) within experimental error, identical, whilst that of the C-4 to C-6 linked dimer procyanidin B-5 was much slower. This observation indicates that in the acid-catalysed decomposition of 4-arylflavan-3-ols, such as the natural procyanidins, formation of the carbocation (8) from the protonated intermediate (7) is *not* (in this instance) rate determining. This conclusion was confirmed using the model substrates (3, 4). The measured initial rates of hydrolysis were almost identical and both were marginally slower than that of procyanidin B-2.

The doubly bridged proanthocyanidin A-2 (5) was resistant to hydrolysis under all the conditions examined and this observation is consistent with the earlier failure to degrade the natural product using toluene-α-thiol and acetic acid. Similarly, the thiobenzyl ether (6) underwent very slow acid-catalysed decomposition in contrast to the parent procyanidins. This confirms the view that the thioether degradation products derived from the procyanidins are products of kinetic, as opposed to thermodynamic, control. The effects of variation of pH and temperature on the decomposition of procyanidin B-2 were also systematically examined. The reaction is clearly dependent on pH, the lower the pH the faster the rate. Plots of the log of the rate constant versus pH gave a linear relationship with lines of slope -1.0 indicative of a specific acid-catalysed reaction which is first order in hydrogen ion concentration. The rate of hydrolytic cleavage of procyanidin

B-2 also increased with increasing temperature and at pH 0.55, the data gave typical Arrhenius plots. Finally, it was noted that in all cases after hydrolysis had proceeded for some time (3 - 4 days) an equilibrium position corresponding to around 80% decomposition was reached. This is consistent with the known reverse reaction (Figure 10) which has been employed synthetically /34/. To substantiate this observation it was noted with procyanidin B-2 that addition of molar quantities of (-) - epicatechin, although it did not affect the initial rate, resulted in a measureable shift in the equilibrium position (∿ 60% decomposition, 3 days).

The data presented is thus consistent with a specific acid-catalysed mechanism in which the rate determining step is clearly the protonation of the 'A' (phloroglucinol type) ring of the 'lower' flavan-3-ol unit. In the cases and conditions examined the effect of stereochemical factors is surprisingly small. Most of the observations made in this study and earlier studies can now be interpreted satisfactorily in terms of this mechanism, Figure 10. The clear difference observed in the rate of hydrolysis of dimers of the C-4 to C-8 type (eg. B-2) and the isomeric C-4 to C-6 linked polyphenols (eg. B-5), is however not readily explicable. Empirically it seems probable that the presence of a *para*-phenolic hydroxyl group to the interflavan bond is more effective in assisting protonation at the interflavan bond (and hence decomposition) than an *ortho*-hydroxyl group. Extrapolation of the data obtained for procyanidin B-2 permitted a rough estimate to be made for the hydrolytic rate constant at pH 4.0, such as appertain in beers at 25°C. The value obtained was $K_{obs,4.0} = 6 \times 10^{-6} h^{-1}$.

Model systems were evaluated in the light of this information in relation to the formation of polyphenol-protein hazes. Sterile solutions (acetate buffer, pH 4.1), containing B.S.A. (300 ppm) with (-)-epicatechin, procyanidin B-2 and a polymeric procyanidin from *Crataegus monogyna* (∿ hexamer based on (-)-epicatechin chain extension and termination units) - all at varying concentrations from 50 - 200 ppm - were set up and the turbidity monitored as an index of haze formation. Over a period of 6 - 8 months, solutions of B.S.A. remained translucent and (-)-epicatechin had a negligible effect on turbidity. However, both procyanidin B-2 and the polymeric procyanidin from hawthorn caused a steady increase in turbidity (haze) and in the case of the latter substrate a precipitate - similar to permanent haze from beer - was eventually formed. This evidence, although circumstantial, thus strongly suggests that the formation of permanent haze in beers is correlated with the presence of oligomeric procyanidins.

Under the weakly acidic conditions prevailing in these beverages, slow decomposition occurs with the initial formation of carbocation species such as (8), Figure 10. In the presence of protein, it is postulated, these may then be captured by nucleophilic species on the protein, such as - SH groups, thus modifying the hydrophilic surface of the protein, resulting in aggregation and finally precipitation. Thioether adducts such as (9), although themselves acid-labile, would be expected to have greater stability than the parent procyanidins in the acidic media, Table 1. It is interesting to note that in acetate buffer solutions at pH 4.1, in the absence of B.S.A., procyanidin B-2 and the oligomer from hawthorn both gave fine brick-red precipitates (phlobaphens) which result presumably from decomposition and polymerisation of the intermediate carbocation (8) Figure 10.

Given the mild conditions under which this type of acid-induced decomposition of procyanidins may occur, it seems entirely reasonable and predictable that analogous interactions with proteins and other macromolecules to give covalently bound complexes of polyphenols may occur in other situations - for example, the microbial decomposition of plant tissues during their incorporation into soils and the acid-mediated breakdown of polyphenol-containing materials in the stomach of ruminants and other animals. Similarly, acid-catalysed decomposition of procyanidin type polyphenols (such as have been described above) may play a significant part in the covalent binding of polyphenols to collagen which occurs during the traditional protracted forms of vegetable tannage practised in earlier times in the conversion of animal hides to leather.

In the light of these observations it is also pertinent to finally comment upon the natural occurrence of plant pro (antho) cyanidins, since the quinone-methide (10) or its protonated derivative, the carbocation (8), have been implicated by numerous workers as intermediates in their biosynthesis in plants. During earlier investigations two groups of pro (antho) cyanidins have been regularly encountered which clearly fall within the category (a) 'those which are insoluble in water and the usual organic solvents' of the original classification of the Robinsons /35/. These are, respectively, the residual apparently insoluble forms remaining in plant tissues after repeated solvent extraction (eg. - methanol → acetone → acetone/water → DMF) and those which are frequently found in plant gums and exudates (eg. *Butea frondosa.*) The former group are, in our experience, indeed characteristic of all pro (antho) cyanidin tissues such as stem, twig, leaf and fruit where they invariably predominate over the more freely soluble oligomeric forms - typically by as much as 5 or 10:1 based on the release of anthocyanidin with acid. Present evidence strongly suggests that these pro (antho) cyanidins are covalently bound to a (poly) saccharide matrix within the plant. Support for this view comes from the analysis of several acetone-water soluble oligomeric pro (antho) cyanidins - for example that isolated from hawthorn after chromatography on Sephadex LH-20 (with Zhaobang-Shen). Degradation with toluene-α-thiol in acetic acid gave residual material of a carbohydrate character in the aqueous soluble fraction. The ^{13}C FT NMR spectrum of the polymer gave a complex pattern of signals, and detailed analysis was not possible, although the broad general pattern of absorptions was, by analogy, consistent with a procyanidin polymeric structure. However, signals at δ 102.5 - 105.4 ppm (-CH) and δ 60.0 - 63.0 ppm ($-CH_2$) are not readily accommodated within such a structural proposal; they are nevertheless consistent with the presence of pyranose sugar residues within the heterogeneous polymeric structure. The former signals may be attributed to the anomeric carbon atom of glycosides and the latter to C-6 of pyranose sugars. Proanthocyanidins generally occur free although some examples are now recorded of the occurrence of the parent flavan-3-ols in glycosylated forms. Whilst non-covalent forces may be involved in the complexation of polyphenol and carbohydrate, it seems eminently reasonable in the light of these observations that such proanthocyanidins may well derive from the adventitous capture of the flavan-4-yl carbocation (or its equivalent 8) during the biosynthesis by hydroxyl groups of saccharide structures (11) in the plant cell, Figures 3, 10. The major biosynthetic thrust in the majority of plants appears to be towards the formation of these heterogeneous polymeric forms (Haslam /36/) and this does raise the question whether these metabolites have a function which is principally structural in nature.

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RECENT ADVANCES IN THE CHEMISTRY, STEREOCHEMISTRY, SYNTHESIS AND PHARMACOLOGY OF FLAVANOLIGNANS

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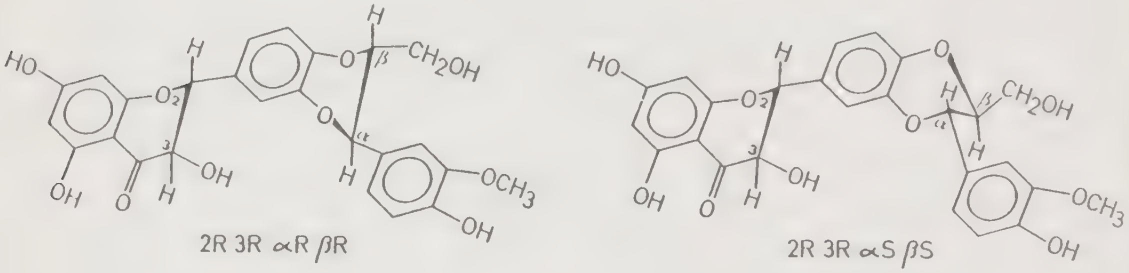
INTRODUCTION

Since the last review article on liver protective agents from plants /1/ some progress has been made, especially in the field of the chemistry and pharmacology of flavanolignans. In order to get more insight into the mechanisms of action and structure-activity relationships, we have concentrated our efforts on the screening and isolation of new naturally occurring flavanolignans, their stereochemistry, synthesis and mode of action on the molecular biological level.

The antihepatotoxic or liver protective constituent of Silybum marianum, silymarin, is a mixture consisting chiefly of silybin (1), silydianin (2) and silychristin (3). In a systematic study of more than a hundred populations of Silybum species /2/ it was found that the pattern of constituents in the seeds of a white-blooming variety of Silybum marianum differed markedly from that of the violet-blooming type. This led to the isolation of two new compounds, silymonin (5) and silandrin (6) /3/, which were then identified as 3-deoxy-silydianin and 3-deoxy-isosilybin /4/. The product of the direct methylation of silandrin was identical with an independently synthesized chalcone tetramethyl ether, thus proving silandrin to be a member of the isosilybin series. The substituted benzdioxanaldehyde reaction partner was synthesized from americanin A monomethyl ether /5/.

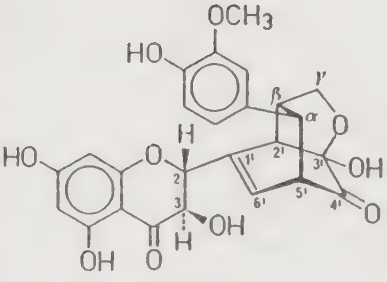
In the meantime, three further new flavanolignans have been isolated from the seeds of the white-blooming plant /6/, using mainly HPLC- and preparative HPTLC-methods. All three compounds silyhermin (7), neosilyhermin A (8) and neosilyhermin B (9) are 3-deoxy-flavanones. Silyhermin is identical with 3-deoxy-silychristin. The other two are diastereomers of a 3-deoxy-isosilychristin, in which the coniferyl alcohol residue, in contrast to silychristin, is linked to the phenyl ring at C_1', C_2', with formation of a dihydrobenzofuran structure.



2R 3R αR βR

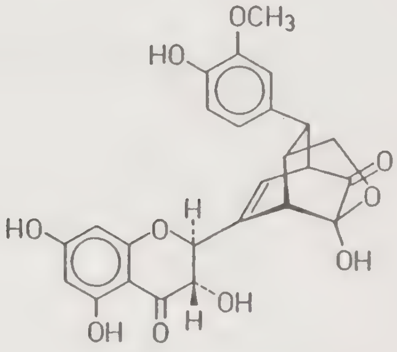
2R 3R αS βS

(+)Silybin AB (1)



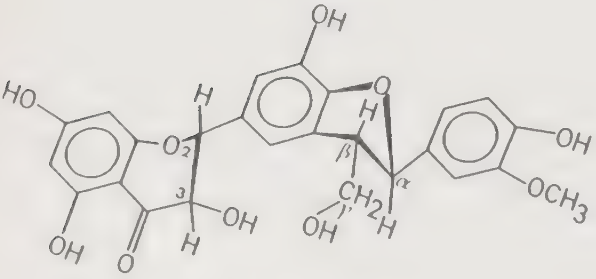
2R 3R αR βR

(+)Silydianin (2)



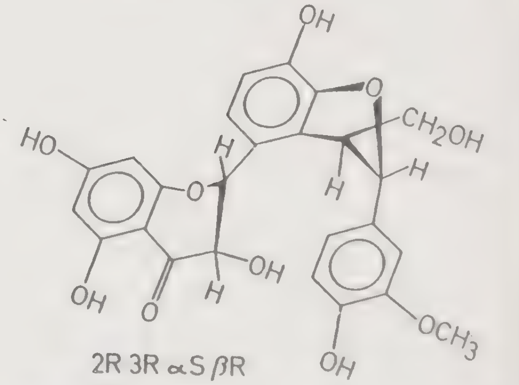
2S 3S αS βS

(-)Silydianin



2R 3R αR βS

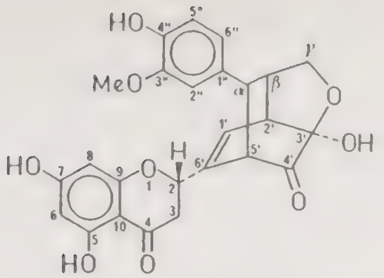
(+)Silychristin (3)



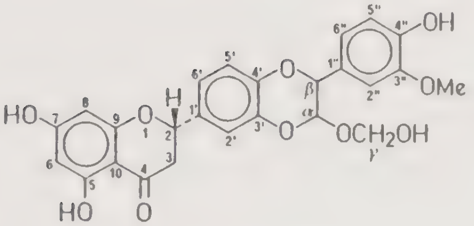
2R 3R αS βR

oder 2R 3R αR βS

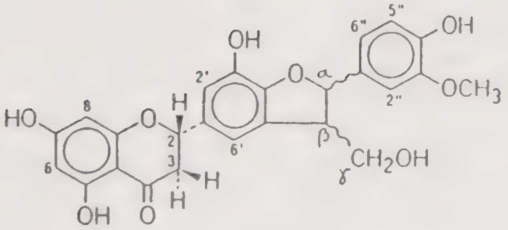
(+)Isosilychristin (4)



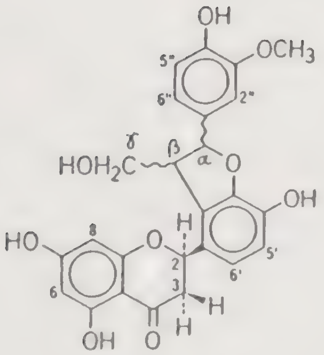
(+)Silymonin (5)



(-)Silandrin (6)

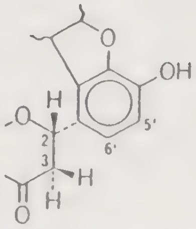


Silyhermin (7)



Neosilyhermin A

(8)



Neosilyhermin B

(9)

STEREOCHEMISTRY (see table 1)

The stereochemistry of the known flavanolignans has been elucidated by Seligmann, Lotter and Wagner /7,8,9,20/, using CD-measurement, NMR spectroscopy and X-ray structural analysis. Oxidative coupling of coniferyl alcohol with the precursors taxifolin or eriodictyol in the course of biosynthesis /10, 11,12/ could theoretically give rise to several possible stereochemical structures of the flavanolignans. The silymarins possess chiral centres in the C-ring (C-2, C-3) and D-ring (C-α , C-ß).

HPLC separation of silybin (1) shows it to be a diastereomeric mixture /13/. X-Ray structural analysis /9,14/ confirmed the half-chair conformation of the benzdioxan ring with *diaxial* H (*J*=8Hz in ^{1}H-NMR) /15/. Positive CD at 330 nm shows 2*R*3*R* configuration /16/. Since the chirality of ring C remains unchanged, but varies in ring D, a 1:1 diastereomeric pair is present with the partial racemic structure α*R*ß*R* and α*S*ß*S*. Naturally occurring 2,3-dehydrosilybin /17/ is accordingly a racemic mixture.

According to Merlini /18,19/, the regioisomeric *isosilybin* also behaves as a 1:1 diastereomeric mixture with analogous configuration.

The structure and relative configuration of silydianin (2) was elucidated in 1970 by X-ray crystallography /20/. Since the positive CD at 345 nm again indicates that the chromanone system has 2*R*3*R* configuration, it can be concluded that the absolute configuration of the bridge C-atoms of the three-ring system is α*R*ß*R* /17/.

On the basis of NMR spectra and chiroptical measurements, the stereochemistry of the 3-deoxy analogue, silymonin /21/, corresponds to that of silydianin, and is therefore 2*S*α*R*ß*R* /7/.

The flavanone compound, silandrin (5) belongs to the isosilybin series /5/. Dehydrogenation of silandrin produces optically active 2,3-dehydrosilandrin, which shows a positive CD for the benzdioxan moiety; the possible configuration is therefore α*R*ß*R* or α*S*ß*S* /7/. Silandrin gives two components in HPLC, and its naturally occurring 2,3-dehydro derivative, *hydnocarpin*, is optically active /22/. The presence of a diastereomeric mixture, as in the case of silybin and isosilybin, but in unequal proportions (2:1), therefore seems probable.

Silychristin (3) has a phenylcumaran structure /23/ and can be predicted to have αβ-*cis* or *trans* substitution. These two possibilities cannot be distinguished a priori on the basis of the α,β-coupling constant. Detailed NMR studies of the similarly structured neolignans /24,25,26/, however, permit the differentiation of *cis*- and *trans*-substituted aryl-dihydrobenzylfurans by their shielded γ- and unshielded α-protons. Silychristin (Hα 5.60 ppm; $^3J_{\alpha\beta}$ 6Hz) is accordingly a *trans* compound, whose $^3J_{\alpha\beta}$ coupling constant of >3Hz provides convincing evidence for a *pseudoequatorial* guaicyl residue and envelope conformation. According to Zanarotti et al. /26/ (+)-2,3-dehydrosilychristin, produced by mild dehydrogenation of silychristin, has a positive CD at 290 nm; silychristin therefore has α*R*β*S* configuration. The 2*R*3*R* configuration of the C-ring can again be deduced from the positive CD of the phloroacetophenone chromophore. In addition to silychristin, small proportions of other stereoisomers are detectable by HPLC. The relatively small coupling constants ($J_{\alpha\beta}$=2Hz) of isosilychristin (4) /27/ indicate presence of *pseudoequatorial* protons, i.e. *pseudoaxial* aryl and methylene substituents. The positive CD at 330 nm shows that the 2*R*3*R* chromanone moiety is again dominant. There are therefore two possible enantiomeric partial structures, corresponding to two possible diastereomers with a relative configuration of α*S*β*R* or α*R*β*S*.

The three most recently isolated isomeric flavanone silymarins /6/ possess a dihydrobenzofuran ring system, and according to the NMR data they are structural analogues of silychristin or isosilychristin, with the corresponding αβ-conformation. Evaluation of the dichrograms revealed a 3-deoxy-silychristin (7) and a 3-deoxy-isosilychristin (8), both with $2S\ r^{*}\ \alpha R\beta S$ configuration. Surprisingly, the CD curve of the third isomeric neo-deoxy-isosilychristin (9) showed a negative maximum at 330 nm. It is therefore the only flavanolignan known so far with 2*R* configuration. Since it is not the enantiomer of 3-

Table 1. Stereochemistry of

Compound	Linkage	Conformation
		B-ring
Silybin (1)	3′-O-α 4,-O-β	2, 3-trans pseudo eq.
Isosilybin	4′-O-α 3′-O-β	"
Silydianin (2)	5′-C-α 2′-C-β 3′-C-γ-O	"
Silychristin (3)	4′-O-α 5′-C-β	"
Isosilychristin (4)	3′-O-α 2′-C-β	"
Silandrin (5)	4′-O-α 3′-O-β	pseudo eq.
Silymonin (6)	5′-C-α 2′-C-β 3′-O-γ-O	"
3-Deoxy-silychristin (7) (Silyhermin)	4′-O-α 5′-C-β	"
3-Deoxyso-silychristin (8) (Neo-Silyhermin A)	3′-O-α 2′-C-β	
Neo-3-deoxyso-silychristin (9) (Neosilyhermin B)	3′-O-α 2′-O-β	"
2, 3-Dehydrosilybin	3′-O-α 4′-C-β	-
3-Dehydro-silychristin	4′-O-α 5′-C-β	
Hydnocarpin	4′-O-α 3′-O-β	-

the flavanolignans

Conformation	Configuration		Components
C-ring	C-ring	D-ring	
α, β-trans eq.	2R3R 2R3R	αRβR αSβS	2(1:1)
"	"	"	2(1:1)
α, β-trans tricyclic	2R3R	αRβR	1
α, β-trans pseudo eq.	2R3R	αRβS	1(+2)
α, β-trans pesudo ax.	2R3R	r* αRβS	?
α, β-trans eq.	2S	αRβR αSβS	2(2:1)
α, β-trans tricyclic	2S	RβR	1
α, β-trans pseudo eq.	2S	r* αRβS	1
α, β-trans pseudo ax.	2S	"	1
"	2R	"	1
α, β-trans eq.	–	αRβR αSβS	Racemic
α, β-trans pseudo eq.	–	αRβS	?
α, β-trans eq.	–	αRβR αSβS	Racemic

deoxy-isosilychristin, and has the same conformation, it must also have the same chirality. It is therefore a diastereomer of 3-deoxy-isosilychristin with 2*R* r^{*} α*R*β*R* configuration.

SYNTHESIS

Several attempts have been made to synthesize these flavanolignans. The first approach, a 15-step synthesis, involved the coupling of a 2-phenyl-benzo-1,4-dioxan aldehyde derivative with a corresponding substituted acetophenone derivative /32/. However, as shown by Merlini et al. /19/, the resulting final product was regioisomeric isosilybin rather than silybin. These authors also reported a simple biomimetic synthesis from 2*R*, 3*R*-dihydroquercetin and coniferyl alcohol in an oxidative phenol coupling reaction, using Ag_2O as the oxidizing agent. This procedure leads to a 6:4 mixture of silybin and isosilybin (Fig. 1). Recently a third synthesis was developed. As in the synthesis of silandrin, the starting material is americanin A, a lignan derivative from the seeds of *Phytolacca americana* /32/. OsO_4-degradation of the monomethyl ether of americanin to a benzdioxanaldehyde derivative, and its subsequent coupling with the appropriate acetophenone yielded a chalcone, which was converted into a flavanolignan (Fig. 2). Using this method, silandrin and hydnocarpin were demonstrated to be members of the isosilybin series /5/ (Fig. 1, Fig. 2).

STRUCTURE-ACTIVITY RELATIONSHIPS

In earlier investigations of silybin, silydianin and silychristin, the three flavanolignans of *Silybum marianum* (violet variety), it was shown that silybin had the highest protective activity in the CCl_4 liver damage model. Since all subsequent animal studies were performed exclusively with silybin or the isomeric mixture (silymarin), structure-activity comparisons between individual isomers were not possible. Comparative measurements were made, however, by Sonnenbichler /33/, using the RNA synthesis model with isolated liver cell nuclei and, in collaboration with Hikino /34/, using the hepatocyte model system /35,36/. In the first system, the increase of RNA synthesis is measured by determination of the

Synthesis of Silybin and Isosilybin

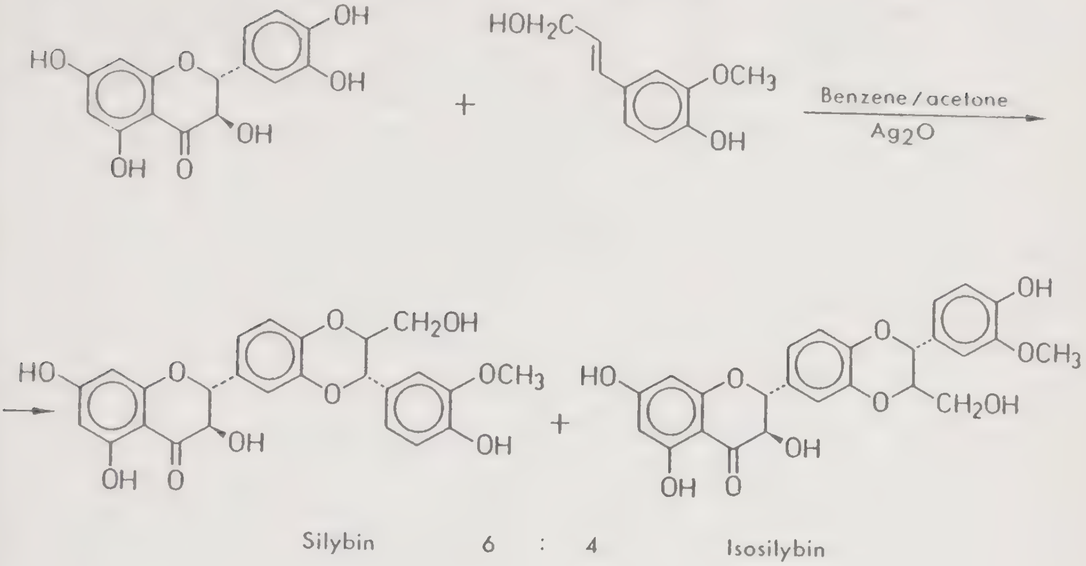


Fig. 1

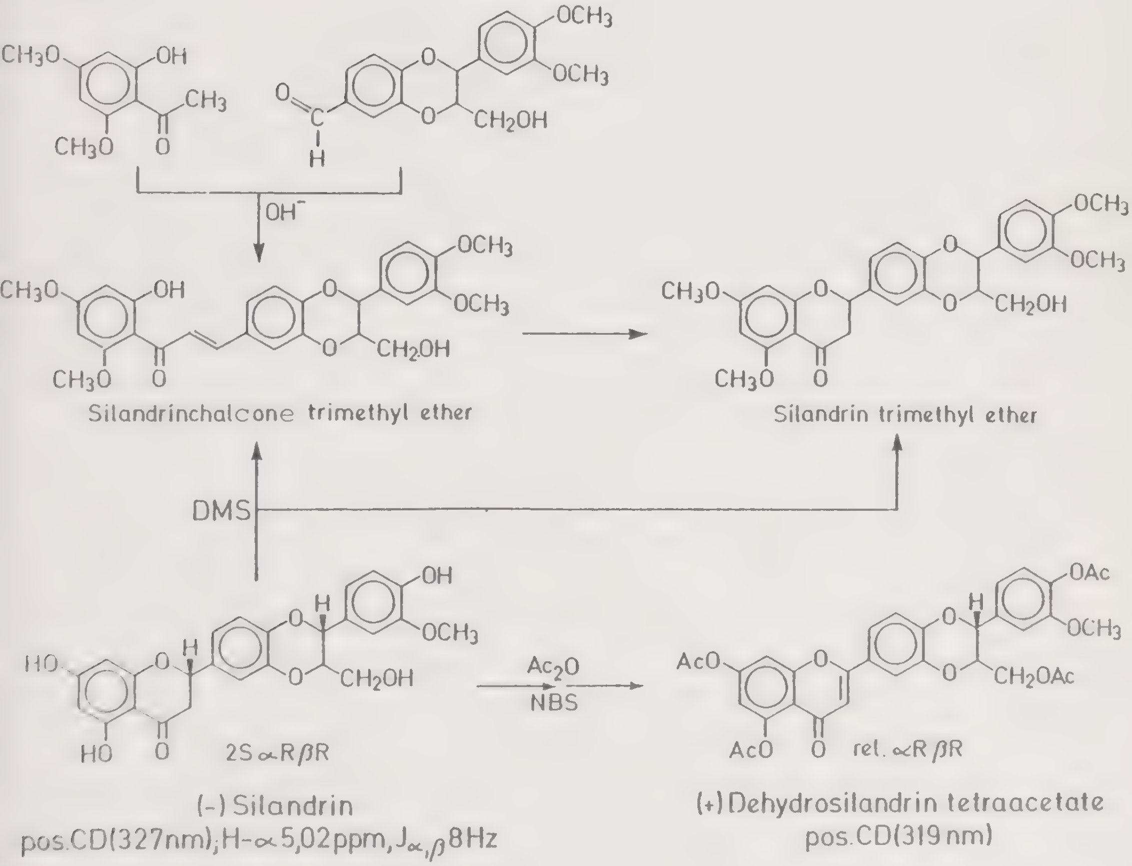

CH$_3$O
OH
CH$_3$
O
OCH$_3$
OCH$_3$
CH$_2$OH
H
OH$^-$
Silandrinchalcone trimethyl ether
Silandrin trimethyl ether
DMS
HO
OH
OCH$_3$
CH$_2$OH
HO
2S αR βR
Ac$_2$O
NBS
AcO
OAc
CH$_2$OAc
rel. αR βR
(-) Silandrin
pos.CD(327nm); H-α 5,02ppm, J$_{\alpha,\beta}$ 8Hz
(+) Dehydrosilandrin tetraacetate
pos.CD(319 nm)

Fig. 2

rate of incorporation of ^{3}H-UTP into various species of liver RNA. In the second model the results of the animal experiments were confirmed. At a concentration of 1.0 mg/ml, silybin shows the highest protective activity against CCl_4 cytotoxicity. In the same system, silychristin and silydianin showed only weak activity. It is interesting that the 3-deoxy compounds, and especially silandrin and 3-deoxy-silychristin, also showed appreciable activity. On the other hand, silydianin showed the highest protective activity against galactosamine cell damage. The biosynthetic precursors, taxifolin and eriodictyol, were inactive as protective agents in the CCl_4 model system, whereas eriodictyol, but not taxifolin, showed protective activity against galactosamine cell damage. Of five other flavones tested by Hikino /37/ (quercetin, isorhamnetin, eupatolitin, cirsimaritin, cirsilineol), only quercetin and isorhamnetin (1.0 mg/kg) showed weak activity. In the investigation of the incorporation of precursors into RNA in liver cell nuclei and hepatocytes, Sonnenbichler had established that the coniferyl alcohol residue was apparently not absolutely essential for the stimulatory activity of the studied compounds. Out of 35 flavonoids studied, the flavanones, taxifolin and hesperetin, and to a slight extent gossypetin, irigenin and the flavane, catechin, all showed activity. The conspicuous status of flavanolignans in animal experiments and in therapy must therefore be due to their lipophilic nature, which gives improved access to the enterohepatic circulation and more favourable pharmacokinetics /38/.

Amongst the model systems for protecion against liver damage, those based on amanitin or phalloidin toxicity are very important, because these two peptides from Amanita phalloides are the most powerful liver poisons known. The cyclic octapeptide, amatoxin, inhibits the synthesis of messenger RNA by complex formation with eukaryotic RNA-polymerase B, thereby bringing protein synthesis to a halt /39/. Phalloidin, a cyclic heptapeptide, which is ten times less poisonous, binds to the contractile protein, actin, and causes destruction of the hepatocytes /40/. The most active antagonist is the cyclic decapeptide, antamanid, which is also present in the death cap fungus (Fig. 3). At a dose of 0.5-1.0 mg/kg, antamanid protects completely against the toxicity of 2.0 mg/kg phalloidin, providing it is administered 10-15 minutes before the phalloidin /41/.

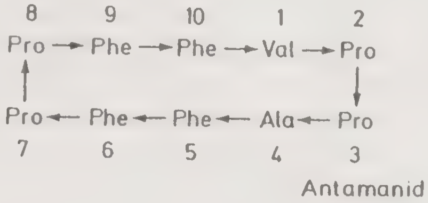


Fig. 3

Antamanid prevents the entry of phallotoxin into the cell interior by competitive binding to a receptor on the cell surface.

Silybin exhibits the same property /42/, but about ten times the amount is required to achieve the same effect /41/. This relatively high receptor specificity of the two compounds suggests, notwithstanding their chemical dissimilarity, that their receptor binding is mediated by similar or identical structural features. For the further investigation of this hypothesis, antamanid bromide was submitted to X-ray structural analysis /43/. This was achieved with the aid of a combination of heavy atom methods, direct methods using an ULTAN- and HELXTL programme, and Fourier cycling of Sim-weighted Fourier syntheses. Comparison of the analysis of antamanid bromide and that of the antamanid-LiBr complex /44/ showed that antamanid is a very flexible molecule with two stable conformations.

According to X-ray structural analysis, silybin exists as a diastereomeric mixture (1:1), in which the flavanone part possesses the 2*R*, 3*R* configuration, and in which Cα, Cß in the benzdioxan moiety assumes both *R* and *S* configurations. As in most flavones, the B-ring of silybin is freely rotatable around the bond axis of ring C. An attempt was then made to find matching regions in the partial structures of both molecules. This was possible by several iteration steps, using a programme by A. Jones on a PDP 11/40 with attached graphic display (Vector General VG 3400) at the computing centre (Fig. 4).

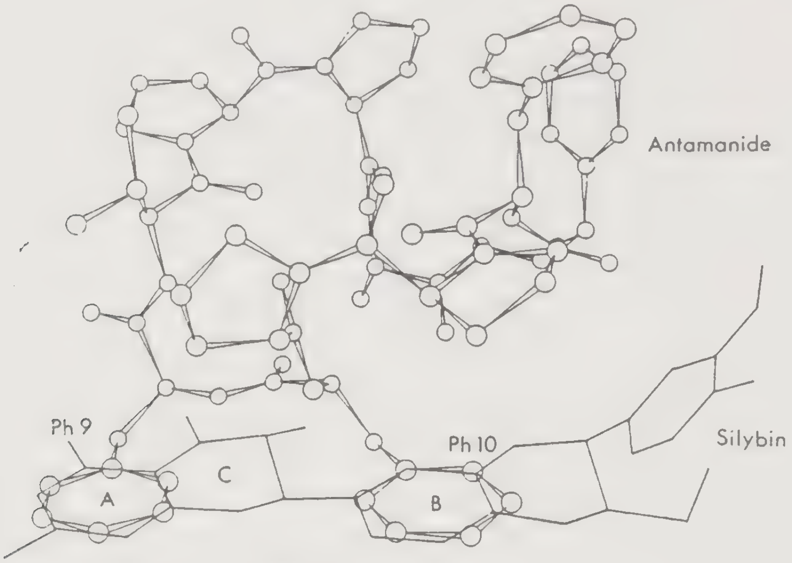
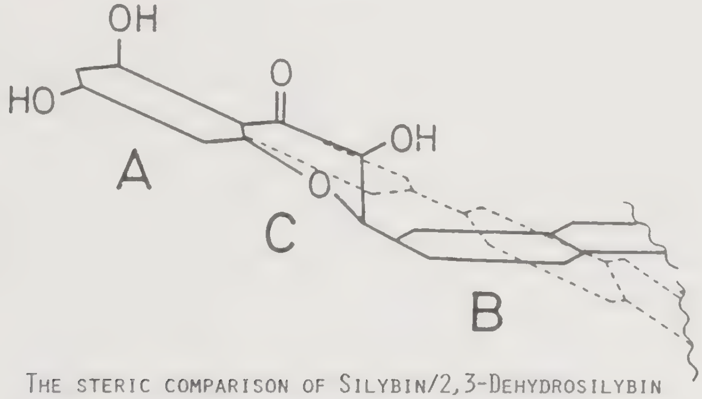


Fig. 4

The coordinates of the complex conformation of antamanid /44/ were used for the comparison with silybin. As seen in Fig. 4, the Phe residues, Phe9 and Phe10, can be rotated around the respective Cα-Cß and Cß-Cγ bonds so that they become aligned in close correspondence with rings A and B of silybin. If it is correct that phenylalanine residues 9 and 10 of antamanid and the aromatic rings A and B of silybin are essential for antitoxicity, then the following prediction can be made. Analogues of antamanid, in which the phenylalanine residues at positions 9 and 10 are replaced by other amino acids, should have very little or no activity. On the other hand, replacements at positions 5 and 6 should have essentially no effect on activity.

As shown by Wieland /41/ this premise is true for some of the 25 synthesized antamanid analogues. Loss of activity was also observed in analogues in which at least one proline is replaced by another amino acid. This is understandable, because the proline residues form four rigid corners to the framework of the cyclic peptide structure. Any replacement of a proline residue must of necessity cause a change in the shape of the peptide backbone, and thereby prevent the attainment of the appropriate conformation.

This has the following implications for the flavanolignans. Whereas the substitution in ring C, e.g. presence or absence of OH on C-3 should have no great effect, dehydrogenation to the 2,3-dehydro compound can certainly be expected to alter the binding properties of the molecule. In the flavanone, there is a small difference in height (∼0.5Å) between rings A and B, and an angle of about 20° between the planes of the two rings. In contrast, the dehydrogenated product must be planar. In 2,3-dehydrosilybin, the positions of the phenyl residues do not correspond so closely with those of the two phenylalanine residues (Fig. 5). In fact, Halbach /45/ has shown that dehydrosilybin has only 35% of the phallotoxin antagonist activity of silybin.



THE STERIC COMPARISON OF SILYBIN/2,3-DEHYDROSILYBIN (DASHED LINE)SHOWS THE DIFFERENT ARRANGEMENT OF THE IMPORTANT RINGS A + B

Fig. 5

Opening of the C-ring appears to have no effect since the α-diketone possesses the same antagonist activity as silybin. Fig. 4 also shows clearly that the benzdioxan moiety itself apparently plays no great part in binding to the receptor. As already postulated, this moiety appears to be important only for transport, i.e. for the appropriate pharmacokinetic properties of the compound.

Diastereometry is also unimportant for receptor binding. From this it can be concluded that the spacing and alignment of the aromatic rings A and B with respect to each other represent the essential structural basis for the anti-phalloidin activity of flavanolignans.

Our next research activity will focus on testing the ability of the proposed receptor model to explain structure-activity relationships in the field of antihepatotoxic flavanolignans.

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Flavonoids and Bioflavonoids, 1985
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FLAVONOID AGLYCONES AS LEAF EXUDATE CONSTITUENTS IN HIGHER PLANTS

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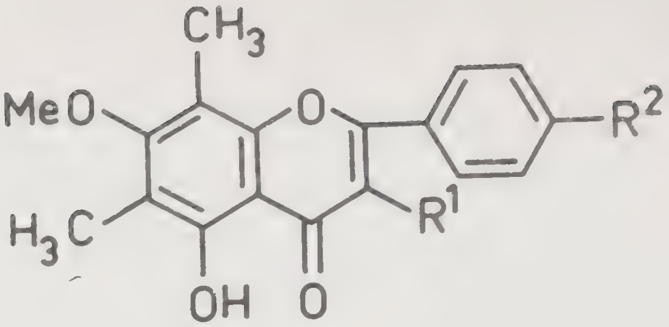
For some 15 years now I have been dealing with flavonoids that are excreted by plants belonging to various taxonomic categories. These continuing studies on flavonoid structures and on their distribution have been very fruitful, as just two examples will demonstrate. The flavones chrysin and tectochrysin were reported from the yellow bud excretion of Populus balsamifera as early as 1873 - we were able, by chromatographic and spectroscopic methods, to show that more than 30 different flavonoids can be present in poplar bud oils /1/. The chalcone-like compound ceroptin was already described in 1903 as major frond exudate component in the fern Pityrogramma triangularis - our studies revealed numerous aglycones to be produced by gymnogrammoid ferns /2, 3/, including many new compounds. In the present paper I want to restrict myself to the occurrence of free flavonoid aglycones in leaf exudates of higher plants and give a short review of our present knowledge on this fascinating subject.

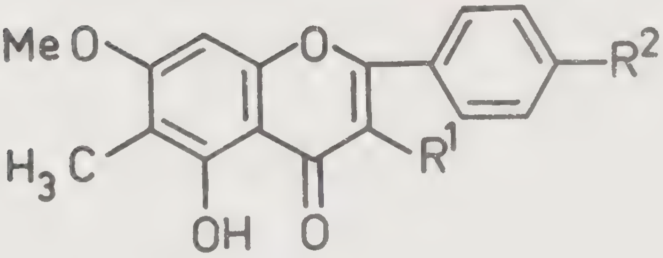
The flavonoids on plant surfaces are probably best known in primroses, some owing their names to this phenomenon, like Primula farinosa and Primula pulverulenta. The white or slightly yellowish powder that is obvious to the naked eye on leaves and inflorescenses is localized in distinct places, as can be seen with a good handlens or a reflected-light microscope. Minute rod- or needle-like crystals cover the head cell of a glandular trichome that excretes this material - we do not yet know in what manner. The mass of densely placed farinose glands brings about the appearance of an uniform farinose layer. This is exactly how the exudate flavonoids occur on fronds of gymnogrammoid ferns as well /4/. Within the higher plants it is exceptional, though, that almost pure mixtures of flavonoid aglycones are accumulated in this way. In many cases they are constituents of resinous coatings on the leaf surfaces. Sometimes these excretions are also obviously produced by glandular trichomes, so for instance in the Californian "sticky monkey flower", Diplacus aurantiaca (lower leaf surface). On many other plants, glands are not obvious, although an important excretion takes place, for instance in Cistus ladanifer which produces the resin ladanum. The amount and nature of the excretion causes the stickiness of its leaves. Another well-known occurrence of flavonoid aglycones is in the wax covering the leaves of Eucalyptus species, where even the scanning electron microscope does not reveal the existence of glands. It must be assumed that in such cases the flavonoids are extruded by the epidermal cells, as is known for waxes /5/. According to our recent studies flavonoids are also encountered where absolutely nothing points to their presence, except perhaps a shiny appearance of the leaf surface that indicates the existence of a thin epicuticular layer.

Epicuticular material often consists of terpenoids, such as ursolic acid, oleanolic acid, etc. In general, terpenoids also form the major part of the resinous excretions: sesquiterpenes and diterpenes have been found, and at least when these resins exhibit a more or less agreable scent, they also contain monoterpenes. The "usual" constituents of epicuticular layers are also encountered: alkanes, long-chain aliphatic alcohols etc. /4/. As yet we have not encountered flavonoids together with fatty acids, glycerids, or wax esters. While alkanes can probably be regarded as more or less ubiquitous compounds, there seems to exist a correlation between the occurrence of exudate terpenoids and externally deposited flavonoids. I want to stress once more - as I did on several opportunities previously - that much more could be known about the distribution of terpenoids and flavonoid aglycones and their joint occurrence. It is unfortunate that phytochemical studies have been and still are almost always done with "ground aerial parts", without regard to the external localization of these natural products. In the past few years an increasing number of publications have reported free flavonoid aglycones, but almost none mentioned their localization. I would bet that in most cases they occur externally. We have checked this in a number of cases, at least with herbarium fragments, and we almost always found our assumption corroborated. There are, of course, other possibilities for the joint occurrence of terpenoids and flavonoids that I don't want to withhold, for instance in the so-called oil-glands of Rutaceae such as Citrus, or in heartwoods, but here we are dealing with leaf exudates only.

The amounts of flavonoids that are deposited externally vary to a great extent, as does the quantity of exudate material in total. I think we can say that in many cases the exudate makes up about 1 - 3 % of dry leaf material. It is much lower when only thin epicuticular layers are present, e.g. in Kalmia and Escallonia, but it can also reach surprisingly high values. We found resinous material up to 18 % of leaf dry-weight in Dodonaea viscosa, and as much as 28 % in Flourensia resinosa /6/. A recent study on some resinous species of Baccharis yielded exudate amounts from 2.3 % in B. bigelovii to 14 % in B. vaccinoides /7/. Unfortunately the flavonoid portions within the exudates are normally very low and the material available is often not sufficient for complete analysis of individual constituents. However, the use for phytochemical studies of the leaf exudate instead of whole-plant extracts means of course a concentration of the flavonoid aglycones. This is why it is often reasonable to re-investigate plant species analyzed previously by other groups. I want to refer to Baccharis again: since the early 1970s ca. 25 different flavonoid aglycones have been reported from some 30 species (and, incidentally, none of these publications

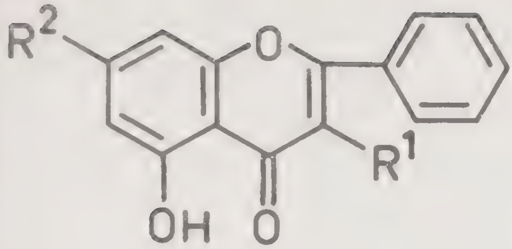


R^1 = H, R^2 = OMe: Eucalyptin
R^1 = H, R^2 = OH: Sideroxylin
R^1 = OH, R^2 = OMe: Kalmiatin
R^1 = R^2 = OH: Latifolin

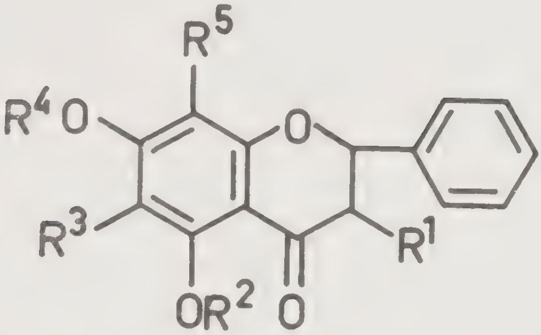


R^1 = H, R^2 = OMe: 8-Desmethyl-eucalyptin
R^1 = H, R^2 = OH: 8-Desmethyl-sideroxylin
R^1 = OH, R^2 = OMe: 8-Desmethyl-kalmiatin
R^1 = R^2 = OH: 8-Desmethyl-latifolin

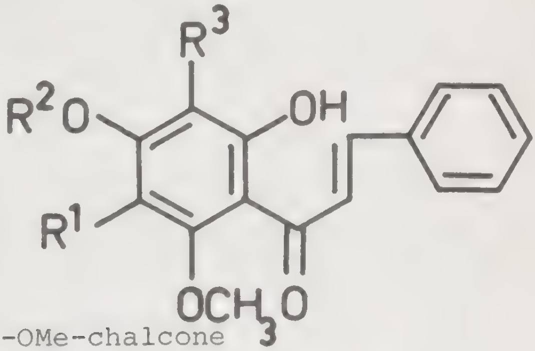
Fig. 1: C-methyl-flavones and -flavonols found in the epicuticular layer of *Kalmia angustifolia* and (in part) in the leaf wax of *Eucalyptus sp.*



$R^1 = H, R^2 = OH$: Chrysin
$R^1 = H, R^2 = OMe$: Tectochrysin
$R^1 = R^2 = OH$: Galangin
$R^1 = OH, R^2 = OMe$: Izalpinin

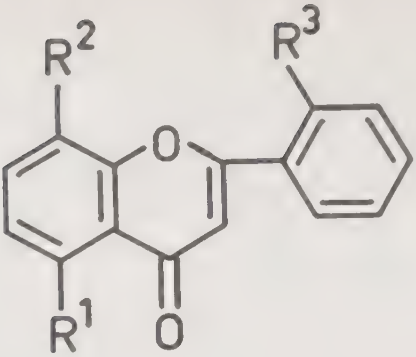


$R^1 = R^2 = R^3 = R^4 = R^5 = H$: Pinocembrin
$R^1 = -O-CO-CH_3, R^2 = R^3 = R^4 = R^5 = H$: Pinobanksin-3-acetate
$R^1 = R^2 = R^4 = R^5 = H, R^3 = CH_3$: Strobopinin
$R^1 = R^4 = R^5 = H, R^2 = R^3 = CH_3$: Strobopinin-5-Me (comptonin)
$R^1 = R^2 = R^3 = R^4 = H, R^5 = CH_3$: Cryptostrobin

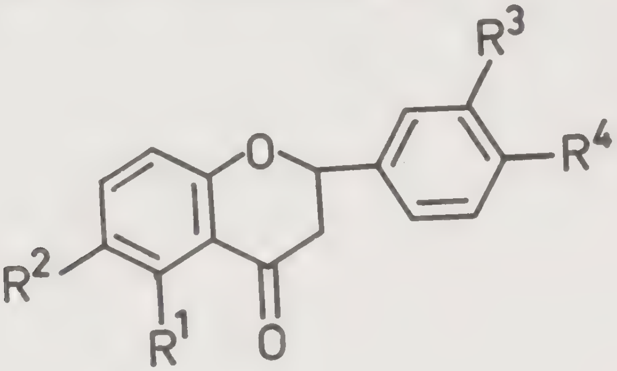


$R^1 = R^2 = R^3 = H$: 2',4'-diOH-6'-OMe-chalcone
$R^1 = CH_3, R^2 = R^3 = H$: 2',4'-diOH-6-OMe-5'-Me-chalcone
$R^1 = R^2 = H, R^3 = CH_3$: 2',4'-diOH-6'-OMe-3'-$CH_3$-chalcone
$R^1 = R^3 = CH_3, R^2 = H$: Myrigalon D
$R^1 = R^2 = R^3 = CH_3$: Myrigalon B

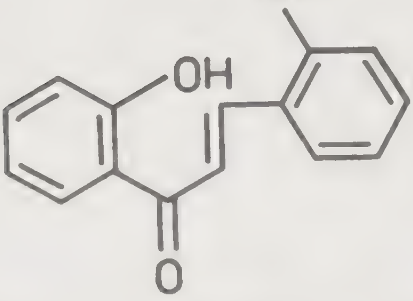
Fig. 2: Flavonoids found in the leaf exudate of Comptonia peregrina



$R^1 = R^2 = R^3$ = H: Flavone
R^1 = OH, $R^2 = R^3$ = H: 5-OH-flavone
$R^1 = R^2$ = H, R^3 = OH: 2'-OH-flavone
$R^1 = R^2$ = H, R^3 = OCH$_3$: 2'-OMe-flavone*
$R^1 = R^2$ = OH, R^3 = H: 5,8-diOH-flavone (primetin)
$R^1 = R^3$ = OH, R^2 = H: 5,2'-diOH-flavone
R^1 = OH, R^2 = H, R^3 = OCH$_3$: 5-OH-2'-OMe-flavone*
$R^1 = R^2 = R^3$ = OH: 5,8,2'-triOH-flavone



$R^1 = R^2$ = H, $R^3 = R^4$ = OH: 3',4'-diOH-flavone
R^1 = OH, R^2 = OCH$_3$, $R^3 = R^4$ = H: 5-OH-6-OMe-flavone



2'2-diOH-chalcone*

Fig. 3: The unusual flavones found in the farina of Primula species (The asterisk denotes novel natural products).

mentioned the external localization). We studied 6 species now and found them to produce about 40 different flavonoid aglycones, including several new structures /7/.

It is certainly not accidental that external flavonoids appear mostly together with terpenoids, but is due to their physical properties (especially their solubility). They are not only aglycones, but also mostly methylated compounds, as such more or less lipophilic and dissolved in the terpenoid resin. We sometimes find in one plant the whole series of possible methyl derivatives (normally with the exception of 5-methyl ethers) of a distinct basis skeleton, the range of polarity thus reaching, for example, from 3-methyl-quercetin to 3,7,3',4-tetramethyl-quercetin in Cistus albidus /8/ or from kaempferol to kaempferol-3,7,4'-trimethyl ether and from the 6-methyl ether to the 3,6,7,4'-tetramethyl ether of 6-hydroxykaempferol in Baccharis vaccinoides /7/. Examples from our recent research that illustrate this are compiled in Table 1. The range of basic structures realized in exudate flavonoids is of course much broader. This is demonstrated by Figs 1 - 3. Fig. 1 shows the C-methyl flavonoids found in Eucalyptus leaf wax and/or in the thin epicuticular layer on leaves of Kalmia angustifolia /9/. In Fig. 2 all the flavonoids found in the leaf exudate of Comptonia peregrina have been compiled. The fact that the lipophilic nature of flavonoids must not necessarily be due to methylation is demonstrated by the aglycones present in the "farina" of Primula species /10/ as shown in Fig. 3. Unsubstituted flavone is the major constituent of the farinose exudate in most species. Primetin can sometimes also reach a rather high percentage, as for instance in Primula japonica. The other flavones (except for 5,8,2'-triOH-flavone) are also rather lipophilic, due to the low number of hydroxyl groups. Three compounds have been identified only recently, namely 2'-methoxy-flavone, 5-hydroxy-2'-methoxy-flavone and 2',2-dihydroxy-chalcone. Their structural elucidation, in particular that of the novel natural chalcone, will be described elsewhere /11/.

Primula is also perhaps the most striking example for the fact that in most plants studied the flavonoids found in the leaf tissue as glycosides are different from those deposited as aglycones externally. Primroses have been described to contain glycosides of kaempferol, of quercetin and, in yellow petals, of gossypetin /12/. These are flavonols, with 4 - 6 hydroxyl groups. Externally in the farina we find flavones, with a maximum of 3 hydroxyl groups, but this latter compound (5,8,2'-triOH-flavone) occurs only in trace amounts, whereas the major component has no OH at all. Who can tell how and where these non-polar compounds are built by the plant? It can be assumed that they are formed in the glandular cells - but from what precursors? I must admit that I don't know the answer to this intriguing question that concerns me ever since I started working this subject. I am still hoping for an inspiration as to how this problem could be dealt with methodically. It is slight comfort to know that also groups specialized in research on flavonoid biosynthesis seem not to engage themselves in this problem, maybe for the same reason. - We have made it routine in our laboratory to check plant material for tissue flavonoids after removal of the exudate by rinsing with organic solvents. After hydrolysis of the methanolic extract we often find trivial aglycones like apigenin, luteolin, kaempferol, quercetin. Their glycosides may exhibit different profiles, but these are not comparable in their variability and specifity with those realized in exudate flavonoids. In other words:the diversity of external aglycones normally exceeds that of the tissue glycosides. This has been demonstrated recently for instance for Ericameria, Hazardia and Heterotheca /12/. We suggest that future studies on such resinous genera should focus on the flavonoid aglycone patterns found in the exudate as opposed to the internal glycoside derivatives /cf. 13/. From this latter sentence appears also the cloven foot of this method: it can only be applied where leaf exudates are present. This limits its practical usefulness to certain families and/or genera and, what is worse, it can even limit its practicability within a genus to those

Table 1: Flavonoid aglycones found in the leaf exudate of some Compositae.

Baccharis bigelovii: Chrysin, apigenin, luteolin, lut-3'-Me, lut-4'-Me; galangin, kaempferol, kae-7,4'-diMe, quercetin, qu-3'-Me; pinocembrin, pinobanksin.

Baccharis sarothroides (Pop. A): Kaempferol, kae-3-Me, kae-3,7-diMe, 6-hydroxykaempferol-3,6-diMe, 6-OH-kae-3,6,7,-triMe, herbacetin-3,8-diMe, "6,8-dihydroxykaempferol"-3,6,8-triMe and -3,6,7,8-tetraMe.

Baccharis sarothroides (Pop. B): Kaempferol, kae-3-Me, kae-3,7-diMe, 6-hydroxykaempferol-3,6-diMe, herbacetin-3,8-diMe, "6,8-dihydroxykaempferol"-3,6,8-triMe.

Baccharis vaccinoides: Apigenin-7-Me, ap-4'-Me, ap-7,4'-diMe, scutellarein-6-Me, scut-6,7-diMe, scut-6,4'-diMe; kaempferol-3-Me, kae-7-Me, kae-4'-Me, kae-3,7-diMe, kae-3,4'-diMe, kae-7,4'-diMe, kae-3,7,4'-triMe, 6-hydroxykaempferol-6-Me, 6-OH-kae-3,6-diMe, 6-OH-kae-6,7-diMe, 6-OH-kae-6,4'-diMe, 6-OH-kae-3,6,7-triMe, 6-OH-kae-3,6,4'-triMe, 6-OH-kae-3,6,7,4'-tetraMe /7/.

Ericameria latifolia: Apigenin, ap-7-Me, ap-4'-Me, ap-7,4'-diMe, luteolin, 6-hydroxyluteolin-6-Me; kaempferol, kae-3-Me, kae-7-Me, kae-4'-Me, kae-3,7-diMe, kae-3,4'-diMe, kae-3,7,4'-triMe, quercetin, qu-3-Me, qu-3'-Me, qu-3,7-diMe, qu-3,3'-diMe, qu-3,7,4'-triMe /13/.

Flourensia resinosa: Chrysin, chrys-7-Me; apigenin, apigenin-7-Me, ap-4'-Me, ap-7,4'-diMe; galangin, gal-3-Me, gal-7-Me, kaempferol-3-Me, kae-3,7-diMe, kae-3,4'-diMe, quercetin-3,3'-diMe; pinobanksin /6/.

Isocoma tenuisecta: Apigenin, luteolin, lut-3'-Me; kaempferol-3-Me, kae-3,7-diMe, kae-3,4'-diMe, kae-3,7,4'-triMe, 6-hydroxykaempferol-3,6,7-triMe, 6-OH-kae-3,6,7,4'-tetraMe, quercetin-3-Me, qu-3,7-diMe, qu-3,3'-diMe.

Table 1, continued.

Isocoma veneta: Apigenin, ap-7-Me, ap-4'-Me, ap-7,4'-diMe, scutellarein-6-Me, luteolin-3'-Me; kaempferol-3-Me, kae-3,7-diMe, kae-3,4'-diMe, kae-3,7,4'-triMe, quercetin-3-Me, qu-3,3'-diMe, qu-3,7,4'-triMe, qu-3,7,3',4'-tetraMe /27/.

Heterotheca grandiflora (Pop. A): Scutellarein-6-7-diMe, luteolin-7,3'-diMe, 6-hydroxyluteolin-6,7,3'-triMe; kaempferol-3,7-diMe, 6-hydroxykaempferol-6,7-diMe, 6-OH-kae-3,6,7-triMe, 6-OH-kae-3,6,4'-triMe, quercetin-3,7,3'-triMe, quercetagetin-3,6-diMe, queg-3,6,7-triMe, queg-6,7,4'-triMe; eriodictyol-7-Me.

Heterotheca grandiflora (Pop. B): Scutellarein-6,7-diMe, kaempferol, kae-3-Me, kae-7-Me, kae-4'-Me, kae-3,7-diMe, kae-3,4'-diMe, 6-hydroxykaempferol-6-Me, 6-OH-kae-3,6-diMe, 6-OH-kae-6,7-diMe, 6-OH-kae-3,6,7-triMe, 6-OH-kae-3,6,4'-triMe, 6-OH-kae-3,6,7,4'-tetraMe, quercetin, au-3-Me, au-7,3'-diMe, qu-3,7,3'-triMe, quercetagetin-6,3'-diMe, queg-3,6,7-triMe.

Heterotheca psammophila (Pop. A): Kaempferol-3,4'-diMe, kae-3,7,4'-triMe, 6-hydroxykaempferol-6,7-diMe, 6-OH-kae-3,6,7-triMe, 6-OH-kae-6,7,4'-triMe, 6-OH-kae,3,6,7,4'-tetraMe, quercetagetin-3,6,7,4'-tetraMe. Scutellarein-6,4'-diMe, scut-6,7,4'-triMe.

Heterotheca psammophila (Pop. B): Kaempferol-3-Me, kae-3,7-diMe, 6-hydroxykaempferol-3,6-diMe, 6-OH-kae-6,7-diMe, 6-OH-kae-3,6,7-triMe, quercetagetin-3,6,7-triMe /28/.

species exhibiting the phenomenon of flavonoid excretion. I believe, however, that this method has its advantages and its validity despite this obvious handicap.

Years ago I published detailed tables on flavonoid patterns observed in bud excretions of poplars /1/, of birch and of elder /14/, which showed that the flavonoid profiles can be typical for species. Very detailed results are also obtained in the course of our research on farina constituents of gymnogrammoid ferns. As has been shown for members of the genus Notholaena in particular /15/, the flavonoid patterns can typify species, recognized varieties, and even chemical races /16/. Such detailed results are not yet available at present for leaf exudate flavonoids of higher plants, but we have hints that similar results may be expected. One example given earlier was Escallonia /17/. I also refer to recent results with Achillea as presented during this Symposium by K. Valant-Vetschera /18/. Further data, in part unpublished, are compiled in Table 1. (At the Symposium some colour photos of thin-layer chromatograms were shown that nicely illustrate different flavonoid patterns.)

The results with Baccharis sarothroides, Heterotheca grandiflora, and Heterotheca psammophila point to another difficulty in these studies: the flavonoid profiles can vary to a great extent between different populations of a certain species. (A further example was shown at the Symposium by a TLC plate with samples of Dodonaea viscosa from different origins.) This, however, is a general problem of chemotaxonomic work. Many collections of different origin, thus comprising different populations, must be analyzed before we can state whether a certain flavonoid pattern typifies a species or a variety with the same certainty as I can state this in Notholaena already. There is no doubt that a lot of work still has to be done in this field.

Next I want to deal with the distribution of flavonoid aglycones within the higher plants. The phenomenon of flavonoid excretion by leaves - is it generally encountered in higher plants, or is it restricted to certain taxa? In earlier publications I have stressed that the occurrence of flavonoid aglycones is mostly correlated to the existence of secretory structures of any kind, and in the first part of this paper I have also mentioned that they often co-occur with excretions of terpenoid nature. This statement should now be extended in so far as flavonoid aglycones are obviously also present in those very thin epicuticular layers on leaf surfaces that are barely visible to the naked eye and where glandular structures are not observed. To date, however, this has hardly ever been checked and only a few cases are known. Hence emphasis of our knowledge about distribution of leaf exudate flavonoids rests indeed on plants with secretory structures and production of leaf resins. (I want to mention that plants producing a glue-like exudate based on polysaccharides as,e.g., Droseraceae,of course do not excrete flavonoid aglycones.) External localization of flavonoid aglycones on leaves obviously occurs scattered within the angiosperm, as can be seen from the following list of families and genera for which we have reliable information: Bignoniaceae (*Phyllarthron*), Cistaceae (*Cistus*), Compositae (*Achillea*, *Adenostoma*, *Artemisia*, *Baccharis*, *Brickellia*, *Chrysothmanus*, *Ericameria*, *Eupatorium*, *Flourensia*, *Haplopappus*, *Heterotheca* ... *Pluchea* ... *Vernonia*), Dicrastyliaceae (*Newcastelia*), Ericaceae (*Gaultheria*, *Kalmia*), Euphorbiaceae (*Bayeria*), Gesneriaceae (*Didymocarpus*), Hydrophyllaceae (*Eriodictyon*, *Wigandia*), Lamiaceae (*Salvia*), Mimosaceae (*Acacia*), Myricaceae (*Comptonia*, *Myrica*), Myrtaceae (*Eucalyptus*), Pennaceae (*Saltera*), Primulaceae (*Primula*), Sapindaceae (*Dodonaea*), Scrophulariaceae (*Mimulus*), Zygophyllaceae (*Larrea*). It is striking that most genera are in the Compositae, strictly speaking the Tubuliflorae (Asteroideae). This can only in part be ascribed to the fact that Compositae is one of the largest families of flowering plants. There is no doubt that production of resinous leaf and stem exudates and excretion of flavonoid aglycones as correlated therewith centre on this family. This list also indicates that there exists a correlation of the occurrence of flavonoid-containing leaf resins with the habitat. Many of the plants found to accumulate exudate flavonoids come from arid or semi-arid regions as I have pointed out earlier /19/. Much of our plant material has been collected in the Sonoran Desert and in the Chihuahuan Desert in Arizona and Mexico (most Compositae, *Eriodictyon* and *Wigandia*, *Acacia neovernicosa*, *Dodonaea viscosa*, *Larrea* sp.). It recently became evident however, that a more generally valid term should be used to describe the habitats from which the plants originate. The term "xeric habitat" might be more adequate as it would also apply for plants like *Hieracium intybaceum* and some *Achillea* species from the Alps, for *Achillea*, *Artemisia*, *Cistus* from the Mediterranean, *Diplacus aurantiacus* from the coastal region of California,etc.

The fact that the epicuticular flavonoids according to our present knowledge occur only in plants of xeric habitats necessarily leads to considerations about their *raison-d'être*. There is no need to discuss the function of terpenoid excretions in reduction of cuticular transpiration. The flavonoid aglycones' solubility in such material would be the most trivial interpretation for their occurrence in these resins. I believe that they are not produced by the plants as a luxury article, nor must they be regarded as waste products. By their localization on the leaf surface they appear especially suited for protective mechanisms. Phenolic products in general possess antimicrobial activities. In all likelihood the exudate flavonoids therefore play a part as antifungal, antibacterial, antiviral agents. Unfortunately only little research on this aspect has been carried out to date /*cf*. 20/. One would also think that their external localization gives them importance as insect deterrents. Sporadic studies on this subject have been published, concerning for instance *Larrea tridentata* /21/, *Diplacus aurantiacus* /22/, *Parthenium sp.* /23/, *Eriodictyon californicum* /24/ (see also /25/). The lipophilic flavonoids are assumed to penetrate better the hostile organisms' cell membranes. Finally it should be mentioned that epicuticular layers and external flavonoids have been reported to reflect irradiation, thus reducing excessive heating up of the leaves /25/, and to absorb excessive UV light. All the possible functions that are discussed would be of special importance for plants that fight for survival in extreme climatic conditions.

An increasing number of researchers now seem to take an interest in the physiological and ecological functions of exudate flavonoids. Thus there is hope that after a few more years more information will be available. That would still not explain why some plants synthesize two dozen or more different flavonoid structures. But if this diversity is a luxury at all, then it is in favour of phytochemists and chemotaxomonists.

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FLAVONOID COMPOSITION IN SOME IRAQI PLANTS USED IN FOLK MEDICINE

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INTRODUCTION

Medicinal plants represent an important component part of Iraqi traditional system of medicine. It has been developed by Arabic people through centuries of combating against nature and deseases, and vast experience has been accumulated in this respect.Nowadays medicinal plants not only play an important role in the people's health service, but also offer an established basis of searching for new drugs by means of modern scientific methods.

The present paper is an attempt to study crude drugs used in traditional medicine for the presence and composition of flavonoids. The plants chosen for investigation were: Crataegus azarolus L., Myrtus communis L., Prosopis farcta L. and Centaurea phyllocephala Boiss. Our findings so far are reported here.

EXPERIMENTAL

Plant materials were collected in March-April (leaves, herbs,flowers) and in May-June (fruits) of 1980 and 1981 from places in and around Baghdad (Centaurea phyllocephala, Myrtus communis, Prosopis farcta) and at the North of Iraq (Crataegus azarolus). The samples were identified in Botany Directorate, National Herbarium of Iraq. Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy and Pharmacology, Biological Research Centre, Baghdad.

Pharmacological-toxicological screening and isolation, phytochemical identification and standardization of the crude drugs and characteristic compounds were carried out by the usual procedures (1,2) and/or as described in earlier publications (3,4,5,6).

RESULTS AND DISCUSSION

1. *Crataegus azarolus* L. (Rosaceae)

Crataegus azarolus was chosen considering its popularity in the northern and middle parts of Iraq as diuretic, and the advantageous therapeutical experiences with various preparations of *Crataegus monogyna* Jacq. and *Cr. laevigata* Cand. (7). Systematic chemical, toxicological, as well as *in vitro* and *in vivo* pharmacological investigations were carried out with the aim to identify the major biologically active constituents.

The alcoholic extract of leaves, flowers and fruits were selectively purified by standard methods, then partitioned using Sephadex LH-20 column chromatography with acetone and water-methanol mixtures as eluents. For toxicological and *in vitro*, *in vivo* pharmacological tests, the extracts and the characteristic bioflavonoid and procyanidin fractions were used (Fig. 1). The acute parenteral toxicity of *Crataegus azarolus* samples was found to be in the range of 1.0-2.3 g/kg, the oral acute toxicity was 5.5 g/kg.

The effect of extracts was studied on the peripheral blood pressure in anaesthetized rats. They produced dose dependent prolonged hypotension responses of i.v. injected adrenaline, the hypertension responses of carotid occlusion, slightly reduced the tachycardia provoked by adrenaline. On isolated right guinea pig atrium *Crataegus azarolus* extracts produced a clear positive inotropic effect, and abolished the arhythmia induced by high doses of isopronaline. The strongest actions were observed using the young leaves, followed by flowers, fruits of the dark,then of the yellowish cultivars.

A direct relation between the amount of procyanidins and the pharmacological actions was established; the presence of bioflavonoids had an important role in the duration of activity.

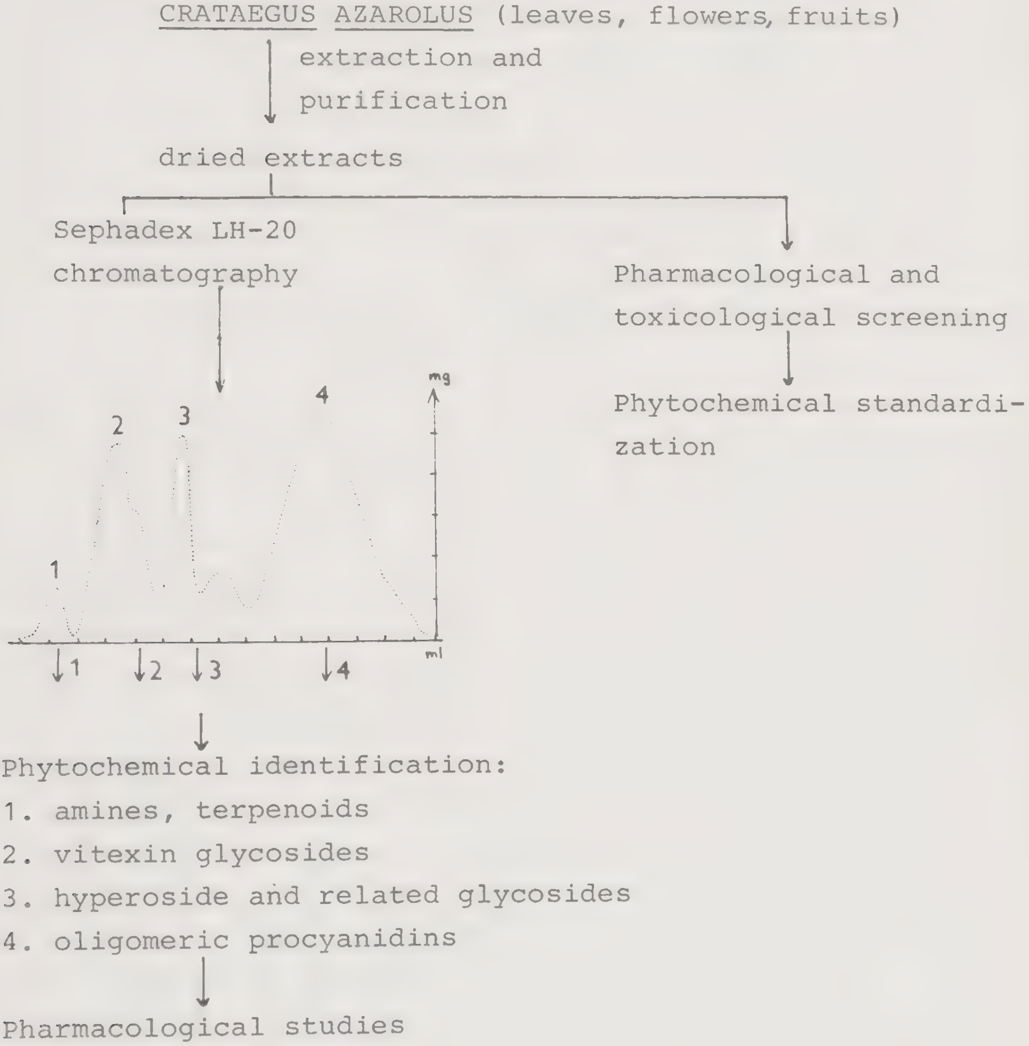
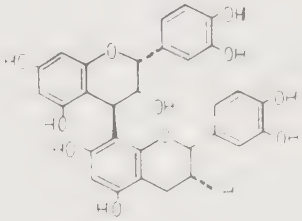

CRATAEGUS AZAROLUS (leaves, flowers, fruits)
extraction and purification
dried extracts
Sephadex LH-20 chromatography
Pharmacological and toxicological screening
Phytochemical standardization
mg
ml
1
2
3
4
1
2
3
4
Phytochemical identification:
1. amines, terpenoids
2. vitexin glycosides
3. hyperoside and related glycosides
4. oligomeric procyanidins
Pharmacological studies

Fig. 1. Flow diagram for the extraction and fractionation of Crataegus azarolus polyphenols

Bioflavonoids were further separated and purified on polyamide columns using water, water-methanol and methanol as eluents. Identification of the major constituents were carried out by comparison with authentic samples isolated from other Crataegus species, using derivatization and UV, IR, ^{1}H-NMR, MS spectroscopic techniques.

The possible use of oriental drugs in medicine was proved besides the toxicological and pharmacological data by the isolation and identification of a series of flavonoids (Table 1) and oligomeric procyanidins (1) (8).



B 2

(1)

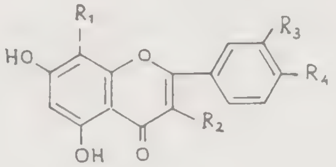
Spectrophotometric methods (9) were used for standardization of the bioflavonoids and procyanidins, and the results are summarized for summitas and fructus of Crataegus azarolus, as percent procyanidins and hyperoside, respectively (Fig. 2).

In agreement with the results of pharmacological tests, both in summitas and fructus drugs the cultivar with dark fruits contains higher percentage of bioflavonoids and procyanidins. It is important to point out that the ratio of leaves, flowers and stems in summitas, and the ratio of fruits and stems in fructus drugs, has a great importance as far the quality of the crude drugs is concerned (Fig. 3).

2. Prosopis farcta L. (Mimosaceae)

About 40 Prosopis species are native in the warmer parts of the world. Most of them are economically important plants of medicinal value, well known in India for their antibacterial properties (10). In Iraq Prosopis farcta L. is widely distributed in agricultural lands and slightly saline places, causing hay fever in humans during flowering, where it is abundant. The

Table 1. Major bioflavonoids isolated and identified in *Crataegus azarolus* grown in Iraq



Compound	R_1	R_2	R_3	R_4
Quercetin	H	OH	OH	OH
Hyperoside	H	O-Ga	OH	OH
Vitexin	Glc	H	OH	H
Vitexin-2"-rhamnoside	Glc-O-Rh	H	OH	H
Orientin	Glc	H	OH	OH

Identification: direct comparison with authentic samples (mp, UV, IR, ^{1}H-NMR, MS), hydrolysis, permethyl derivatives

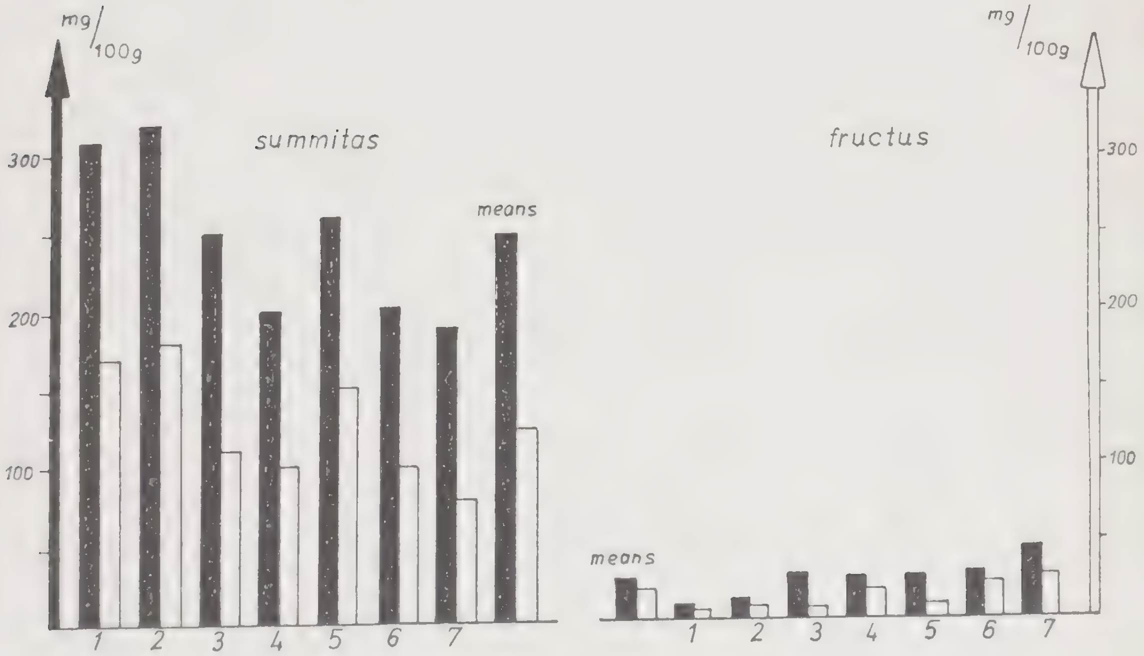


Fig. 2. Procyanidin and bioflavonoid content of *Crataegus azarolus* summitas and fructus

▆ = procyanidins, ☐= flavonoids

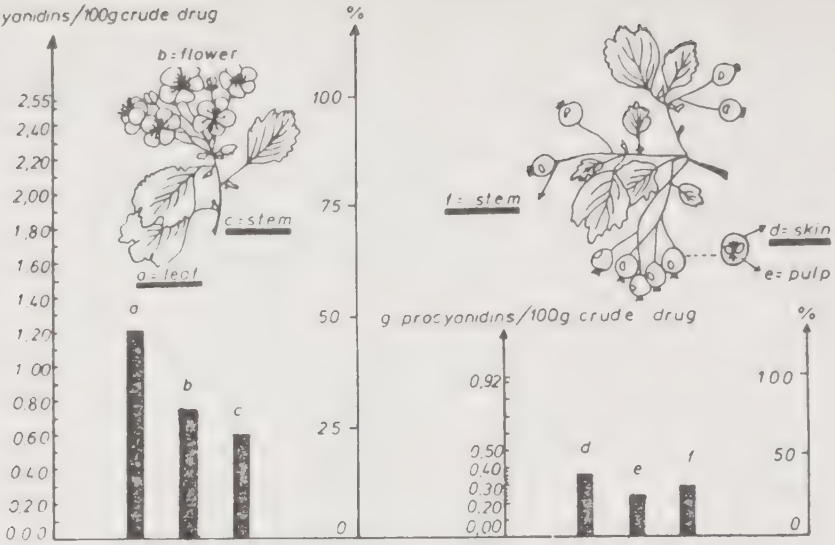


Fig. 3. Distribution of procyanidins in summitas and fructus drugs of Crataegus azarolus

pods are eaten by sheep and camels and have been chewed by people in rural areas in time of scarcity, hence doubtless the name 'tamur al fakira'= poor women's dates (11). There exists the belief that the fresh pods have healing properties and antiviral activity.

From the pods and seeds we have isolated and identified patuletin, patulitrin, rutin, gallic acid by spectroscopic techniques and authentic sample comparison, which are known constituents in other Prosopis species (12). Proanthocyanidins have been separated and identified additively and they were quantitatively characterized, as potentially active molecules.

3. Myrtus communis L. (Myrtaceae)

Decoctions of myrtle branches are being used in indigenous system of medicine as an antimicrobial agent in Mediterranean countries (13,14). Screening of Iraqi plants and their purified extracts for possible antimicrobial and antifungal activities resulted in the isolation of polyphenolic fractions of Myrtus communis leaves, flowers and fruits. The ethyl acetate soluble part of their polaric extract was redissolved and applied to a Sephadex LH-60 column and equilibrated with acetone and methanol. The fractions showing antibacterial activity were further purified on Sephadex LH-20 and Sephadex G-25 columns with acetone, methanol and 50% methanol, respectively (Fig. 4). The process resulted in isolating a less active fraction, containing flavonoids besides tannins (myricetin, kaempferol from fraction B, and myricitrin from fraction A) and a more active hydrolysable oligomeric molecule, whose structure determination should be the aim of further work; nevertheless it was characterized as ellagitannin by chemical reactions, and its constituents were identified after hydrolysis as ellagic acid, gallic acid and glucose using spectroscopic techniques (UV, IR, NMR, MS) and authentic sample comparison.

The antimicrobial assay has been carried out by disc diffusion technique. The clear zone inhibition around the disc was measured in millimeters and compared with those of widely used antibiotics. The ethyl acetate soluble portion and fractions A, B, C were tested, respectively, for the following microorganisms: Gram positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, Gram negative bacteria: *Escherichia coli*, *Pseudomonas aureus*, Fungi: *Candida albicans*, *C. utilis*, *C. tropicalis*.

Activity ratios were calculated for all the samples on the basis of inhibition zones and concentrations of the tested materials. It clearly shows the efficacy of purification for the bioactive constituents (Table 2). LD 50 of the ethyl acetate soluble fraction was 0.52 g/kg.

Table 2. Purification of antibacterial substance from *Myrtus communis*

Fraction	Yield, mg	Activity ratio	Recovery, %
Ethyl acetate soluble portion	1500	1.0	100
Fraction A	650	1.7	74
Fraction B	300	2.5	50
Fraction C	207	4.5	31

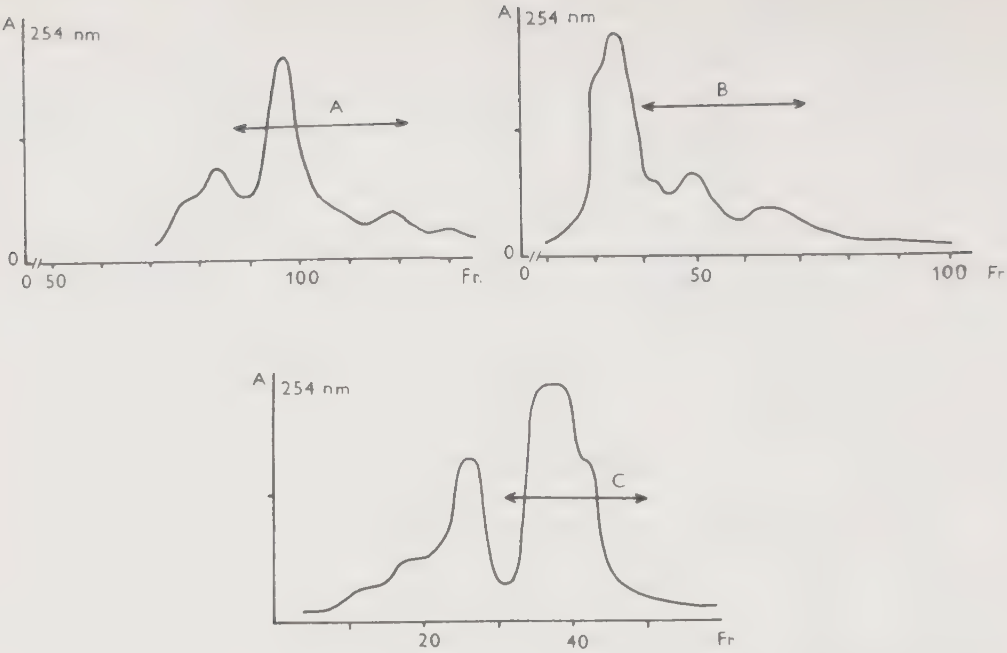


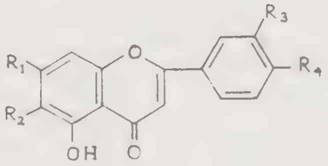
Fig. 4. Purification procedure of antibacterial fractions of the leaves of *Myrtus communis* on Sephadex LH-60 (A), Sephadex LH-20 (B) and Sephadex G-25 (C)

4. *Centaurea phyllocephala* Boiss. (Asteraceae)

Centaurea phyllocephala has been used in folk medicine as an antidiabetic agent. Studies on its various extracts revealed that basal plasma glucose concentration and plasma glucose load were either elevated or unchanged by the administration of the plant extracts. The predominant effect of *C. phyllocephala* is the toxicity in laboratory animals (rats and mice). Acute and chronic toxicity as well as histopathological studies on organs (liver, kidney, spleen, intestine) of the treated and untreated animals strongly contraindicated its applicability as hypoglycaemic agent (6).

Bioassay-directed isolation resulted in five methylated flavonoids. Three of them including hispidulin (5,7,4′-trihydroxy-6-methoxyflavone), nepetin (5,7,3′,4′-tetrahydroxy-6-methoxyflavone) and jaceosidin (5,7,4′-trihydroxy-6,3′-dimethoxyflavone) were identified both in free and glycosidic form, while cirsiliol (5,3′,4′-trihydroxy-6,7-dimethoxyflavone) and 5-monohydroxy-6,7,3′,4′-tetramethoxyflavone were detected

Table 3. Centaurea phyllocephala flavones



	C_7	C_6	C_5	C_3	C_4	
	R_1	R_2	OH	R_3	R_4	
I	OMe	OMe	OH	OH	OH	(cirsiliol)
II	OH	OMe	OH	OMe	OH	(jaceosidin)
III	OH	OMe	OH	OH	OH	(nepetin)
IV	OH	OMe	OH	H	OH	(hispidulin)
V	OMe	OMe	OH	OMe	OMe	

only as aglycones (15). All of the flavonoids were identified by spectral methods (UV, IR, NMR, MS) and authentic sample comparison (Table 3).

CONCLUSIONS

The policy of primary health care, promoted by WHO, operates in making the most rational use of all the local resources and the local medicinal plants.

Present day use of medicinal plants in Iraq covers the following areas: 1. direct consumption of the plant in fresh or dried form, 2. raw materials for the preparation of extracts, tinctures and galenicals.

This work on studying the presence and composition of flavonoids as potentially active agents, adjuvants, or/and characteristic constituents, is an attempt to ensure that plants used in folk medicine are not dangerous and are effective.

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TISSUE-SPECIFIC PRODUCTION OF FLAVONOIDS IN *HORDEUM* AND IN *GLYCINE*

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INTRODUCTION

There is no evidence that flavonoids are essential for the autonomous growth and development of plants. However, there is convincing evidence that flavonoids are important in adapting plants to their environment. These roles include general mechanisms such as absorbing ultraviolet light in the epidermis before it penetrates into the mesophyll, to more specific involvement in the interaction of plants with herbivores, parasites and pathogens /1/. The more specific mechanisms may involve several tissue types. If these ideas are correct, one would predict that flavonoids might generally be found in epidermal cells, while their occurrence in underlying tissues would suggest that they may have species-specific roles in adapting plants to cope with their particular set of herbivores, parasites, and pathogens. Plants have evolved different patterns of flavonoids and they may also have evolved different patterns of distributing these within their epidermis, mesophyll, and other tissues.

Using protoplasts isolated from oat (Avena sativa) leaves, Weissenboeck and his associates have reported that the mesophyll contains flavonoids, synthesizes flavonoids from labeled precursors /2/, and has high activity for several enzymes of flavonoid biosynthesis /3/. However, in pea (Pisum sativum) leaves, Hrazdina et al./4/ find flavonoid glycosides only in the epidermis and neither flavonoids nor enzymes of flavonoid biosynthesis are detected in pea leaf mesophyll.

These contrasting patterns of flavonoid distribution within oat and pea leaves may indicate fundamental differences in their strategies of defensive chemistry, or they may relate to basic differences in the internal anatomy of cereal and legume leaves. To study this in more detail, we have chosen to investigate tissue-specific distribution and regulation of flavonoid biosynthesis and accumulation in primary leaves of barley and in young trifoliate leaves of soybean.

The flavonoid metabolism of the barley primary leaf has been well characterized /6,7/ and its mesophyll protoplasts have been reported to contain activity for at least two key enzymes of flavonoid biosynthesis; Phenylalanine ammonia-lyase /8/ and Isovitexin 7-O-glucosyltransferase /9/.

Soybean leaves normally accumulate kaempferol and quercetin glycosides, and 32 phenotypic classes of soybeans have been characterized based on flavonol patterns in whole leaves /10/. Soybean cultivars which contain the rare glycoside kaempferol 3-(2^G-glucosylgentiobioside) fix CO_2 at rates significantly lower than those lacking this compound, and genetic analysis suggested that the presence of this flavonoid glycoside in the leaf is directly inhibitory to photosynthesis /11/. The inducible isoflavonoid phytoalexins of soybeans have also been extensively investigated /12/, although little is known of their tissue distribution.

This study reports the accumulation of flavonoids and the activity of some enzymes of their biosynthesis in whole leaf and tissue preparations of barley and soybean seedlings.

EXPERIMENTAL

Barley (Hordeum vulgare L. cv Atlas 68) was grown on vermiculite at 22°, while soybeans (Glycine max cv Harsoy 63 and experimental strain OX-913) were grown on a commercial potting mixture at 28°. The seedlings were maintained in culture chambers on a 16/8 h light/dark cycle with 10 klux of cool white fluorescent light and watered daily with Hoagland's nutrient solution.

Primary leaves of barley were harvested five days after planting. A transverse cut was made through the epidermis 1 cm below the apex, and both the upper and lower epidermis were peeled from the subtending 3 cm region. These peels were used to determine enzyme activity and flavonoid accumulation in the epidermis. The peeled leaf pieces were incubated in a protoplast isolating medium containing 20 mM pH 5.8 MES-KOH buffer, 0.5 M sorbitol, 1 mM $CaCl_2$, 0.2% (w/v) bovine serum albumin, 2% (w/v) Cellulysin (Calbiochem, USA) for two hours at 30^o. The digest was filtered through 70 µm nylon mesh which passed the protoplasts but retained the vascular strands. Protoplasts were washed by 50 x g centrifugation for two minutes in medium without cellulytic enzymes.

Soybean mesophyll cells were obtained by cutting 2g of six or 12 day old (see results) first trifoliate leaflets into ca. 1 cm strips and incubating them for 15 min in 20 mM pH 5.8 MES-KOH buffer containing 0.3M sorbitol, 1 mM $CaCl_2$, 2% (w/v) PVP-40, 0.1% (w/v) methyl cellulose, 0.1% (w/v) bovine serum albumin and 2% (w/v) Macerase (Calbiochem, USA). The cells were collected by filtration through a 70 µm mesh. Although these isolated mesophyll cells proved to be highly intact and satisfactory for the present experiments, we have been unable to obtain epidermal peels, or to prepare protoplasts from the epidermis or the mesophyll of soybean leaves.

We have previously described our techniques for TLC and HPLC determination of flavonoids, and for spectroscopic or radioactive measurement of enzyme activity in whole leaf or tissue fractions of these plants /12,13/.

RESULTS

The relative distribution of flavonoids and of several enzymes of phenolic metabolism between the epidermis, mesophyll and vascular strands of five day old barley primary leaves is shown in Table 1. The epidermal peels and vascular strands showed less than 3% contamination by mesophyll cells based on chlorophyll recovery. Approximately 71% of the mesophyll cells were recovered as purified protoplasts.

Table 1 indicates a striking compartmentation of flavonoids within the barley leaf: most of the saponarin is found in the epidermis, all of the lutonarin (3'-OH-saponarin)

Table 1

Tissue localization of flavonoids and enzyme activity in five-day-old barley primary leaves

Parameter	% Distribution between tissues			% Recovery[1]
	Epidermal	Mesophyll	Vascular	
		Flavonoids[2]		
Saponarin	89%	12%	0%	100%
Lutonarin	0%	100%	0%	100%
		Enzyme activity[3]		
PAL	19%	7%	74%	42%
CoA-ligase	0%	89%	11%	55%
CFI	24%	65%	11%	70%

[1] Recovery = sum of flavonoid or enzyme activity measured in epidermal, mesophyll and vascular preparations as % of that in whole leaf segments (calculated per cm^2 leaf tissue).

[2] Whole leaf segments contained 12.8 nmol saponarin and 1.6 nmol of lutonarin per cm^2.

[3] Whole leaf enzyme activity was: PAL (Phenylalanine ammonia-lyase) = 0.74 nmol/min/cm^2. CoA-ligase (4-coumarate CoA ligase) = 0.33 nmol/min/cm$_2$. CFI (Chalcone-flavanone isomerase)= 21.27 nmol/min/cm^2.

is found in the mesophyll, and neither of these flavonoids is associated with the vascular strands. However, the summation of enzyme activity in the tissue preparations was appreciably less than that in equivalent samples of whole leaf material. Other experiments (not shown) indicate that this low recovery of enzyme activity is attributable in part to loss of enzyme activity brought about by incubating the peeled leaves at high osmotic concentrations for two hours in pH 5.8 buffer to obtain mesophyll protoplasts. Scanning electron microscopy also showed that the act of peeling caused extensive fissures in the walls of the epidermal peels. It was not possible to determine the degree to which enzymes may have been lost from mechanically damaged cells of the epidermis, although the recovery of all of the flavonoids from the leaf in the epidermal and mesophyll preparations (Table 1) suggests that the vacuoles of the epidermis, as well as of the mesophyll, remained intact. Accordingly, we conclude that such techniques may serve to demonstrate the presence or absence of enzyme activity within various tissues, but the level of enzyme activity may be appreciably underestimated.

Soybean leaves can not be peeled to obtain epidermal tissues and the mesophyll cell walls are so resistant to fungal cellulases that protoplasts are not obtained in satisfactory yields. However, it is possible to isolate whole soybean mesophyll cells within 15 min, in non-plasmolyzing media, by using pectinases to digest away the middle lamella and free the intact cells into the medium. Using these rapid procedures, we have investigated the distribution of flavonoids and enzymes of flavonoid biosynthesis in a soybean strain in which the accumulation of flavonoid K9 [kaempferol3-(2^G-glucosylgentiobioside)] is closely associated with reduced photosynthetic rates /10/. We have also studied the tissue-specific distribution of isoflavonoid induction in soybean leaves.

The distribution of kaempferol glycosides and enzymes of flavonol bioysnthesis in mesophyll cells isolated from the K9-containing, low-photosynthetic, strain OX-913 of soybeans is shown in Table 2. The only flavonoids detected in this strain are kaempferol glycosides.

To increase the sensitivity of flavonoid detection, methanolic extracts of soybean whole leaflets or isolated mesophyll cells were hydrolyzed for one hour in 1N HCl to convert kaempferol glycosides to the aglycone. Our HPLC analysis can detect kaempferol aglycone at levels below 0.03 µmol/mg Chl. The mesophyll samples were free of detectable kaempferol. Since less than 2% of the kaempferol glycosides could have escaped detection, we conclude that although the kaempferol glycoside K9 may be closely linked with traits that cause low photosynthetic rates, this flavonoid can not be directly inhibitory to photosynthetic reactions, since it is absent from the photosynthetic mesophyll.

Table 2

Distribution of kaempferol glycosides and enzymes of flavonoid biosynthesis in mesophyll cells isolated from OX-913 soybean leaflets at stages of maximal enzyme activity (3 cm long) or flavonoid content (12 cm long)

	Whole leaflet	Cells	% in cells
	Kaempferol glycosides/mg Chl		
K9	0.89 µmol	0	0
Other	0.41 µmol	0	0
	Enzyme activity[1]/mg Chl		
SDH	647 nmol/min	432 nmol/min	70
PAL	2.2 nmol/min	0	0
CoA-ligase	32.7 nmol.min	7.9 nmol/min	24
CFI	7.1 umol/min	2.5 umol/min	35

1 SDH = Shikimate dehydrogenase, PAL = Phenylalanine ammonia lyase, CoA-ligase = 4-Coumarate:CoA ligase, CFI = Chalcone-flavanone isomerase.

Examination of TLC plates from soybean mesophyll preparations disclosed several pale-blue fluorescing (presumably phenylpropanoid) compounds which were not further investigated, although they suggest that the mesophyll was at some stage capable of phenylpropanoid biosynthesis. The absence of PAL from mesophyll cells at this stage of leaf growth suggests that diversion of phenylalanine into phenylpropanoids ceases very early in soybean mesophyll development.

Since soybean mesophyll cells were shown to be normally free of flavonoids, we were interested in determining if they could be induced to form flavonoids. To do this, we isolated mesophyll cells from leaves of soybeans treated with the herbicide Acifluorfen which induces several isoflavonoids (isoflavone aglycones, isoflavone glucosides, pterocarpans and coumestans) in soybean leaves /15/. The tissue distribution of these induced flavonoids has not been previously investigated. The results of that study are shown in Table 3.

Table 3

Acifluorfen-induced flavonoids and enzymes of flavonoid biosynthesis in whole leaflets and in mesophyll cells isolated from Harsoy 63 soybeans.

The leaves of 14-day-old plants were sprayed with an aqueous solution of 100 mg/l Acifluorfen and 0.01% Triton X-100 and samples were taken at the highest stage of flavonoid content or enzyme activity (72 h for flavonoids and IGT, 30 h for PAL and CHS)

	Whole leaflet	Mesophyll cells	% in cells
	Flavonoids (nmol/mg Chl)		
Aglycones			
Daidzein (D)	10	0.4	4.0%
Formononetin (F)	71	3.1	5.2%
Glyceollins	205	7.9	3.1%
Glyceofuran	44	0.5	1.2%
Glycosides			
D-7-O-glucoside	98	25	26.0%
F-7-O-glucoside	16	3.6	22.0%
Kaempferol-gly.	615	9.0	1.4%
	Enzymes (µkat/Kg Chl)[1]		
PAL	24.7	3.5	14%
CHS	6.5	1.3	24%
IGT	94.0	18.0	20%

[1] PAL = Phenylalanine ammonia-lyase, CHS = Chalcone synthase, IGT = Isoflavone 7-O-glucosyltransferase.

Mesophyll cells isolated from control plants had no flavonoids and no activity for PAL, CHS or IGT. Acifluorfen-treated plants formed small lesions on the leaf, but continued to grow and photosynthesize at a normal rate. Mesophyll cells isolated from Acifluorfen-treated leaves (Table 3) had induced activity for PAL, CHS and IGT, and accumulated isoflavone glucosides. Only traces, presumably as contaminants, of other flavonoids were found in the induced mesophyll cells.

From these results with soybeans, we suggest that; 1) Acifluorfen treatment induces enzymes of flavonoid biosynthesis in the mesophyll of expanded soybean leaves, 2) isoflavone glucosides are accumulated in intact mesophyll cells as soluble detoxification products and, 3) isoflavone aglycones and pterocarpans accumulate in the epidermis or extracellularly within the mesophyll.

DISCUSSION

Recent investigations in other laboratories have shown that flavonoid metabolism is restricted to epidermal tissues in legumes such as peas /4/, while cereals such as oats synthesize and accumulate flavonoids in both epidermal and mesophyll tissues /2,3/. Our present work with another cereal (barley) and another legume (soybean) is in agreement with these findings.

Although the presence of flavonoids in the mesophyll tissue of cereals such as oats and barley does not provide information about flavonoid subcellular localization, the absence of flavonoids from the mesophyll of peas and soybeans clearly rules out flavonoid accumulation in mesophyll chloroplasts of those plants. Along these lines, several workers have reported that flavonoids are absent from chloroplasts isolated from peas /4/ but present in chloroplast preparations from oats /16/ and barley /17/. At the least, these results suggest that there is no single pattern of flavonoid distribution between the tissues of plant leaves, and reopens the question of different patterns of subcellular distribution of flavonoids within the plant kingdom.

Our results with barley indicate that mesophyll protoplasts can not be considered metabolically equivalent to mesophyll cells in vivo. It is necessary to maintain an osmoticum of approximately 0.5 M while isolating protoplast in order to keep them from lysing. In oat mesophyll protoplast this degree of osmotic stress induces polyamine biosynthesis /18/ and increases protease activity /19/. Our low recovery of enzyme activity in barley protoplasts (Table 1), may reflect inhibitory effects on enzymes of flavonoid biosynthesis.

Our determination that kaempferol 3-(2^G-glucosylgentiobioside), flavonoid K9, is absent from the mesophyll of soybeans emphasizes the importance of determining tissue distribution of flavonoids in any attempt to explain their possible roles in the plant. Genetic analysis had shown that whenever genes Fg1 and Fg2 were present in soybeans the leaves accumulated this rare kaempferol glycoside /9/. The presence of flavonoid K9 was absolutely correlated with low chlorophyll, low photosynthetic rates, and low seed yields, but fifteen other patterns of kaempferol and quercetin glycosides in soybean isolines had no effect on photosynthetic or yield parameters /10/. Kaempferol has been shown to be a potent inhibitor of photosynthesis in vitro /14/. However, since neither kaempferol or any of its glycosides is accumulated in the mesophyll of soybean (Table 2), this seems to rule out any possibility that flavonoid K9 can have a direct inhibitory effect on soybean photosynthesis.

When plants are subjected to severe stress they commonly show enhanced flavonoid accumulation /20/. In soybean, either fungal attack or treatment with certain chemicals leads to the rapid induction of isoflavonoids /11/ which may serve to protect the plants from plant pathogens /15/. Since fungal pathogens presumably attack the mesophyll as well as the epidermis, we were interested in determining the degree to which the normally flavonoid-free mesophyll might participate in isoflavonoid induction and accumulation. In this work we induced isoflavonoids with the herbicide Acifluorfen whose effects on soybean phytoalexin induction are similar to those of various fungal elicitors /15/.

Acifluorfen effects on soybean flavonoids seem to be remarkably specific for isoflavonoids. This treatment caused no detectable change in the qualitative or quantitative production of kaempferol glycosides of the whole leaf (Table 3), but the total flavonoid content doubled within 24 hours due entirely to the accumulation of isoflavonoids. Acifluorfen also induced activity for chalcone-synthase in the mesophyll and markedly increased the activity of PAL and IGT. Stages of enzyme activity correlated well with rates of product accumulation. PAL produces phenylpropanoid precursors for chalcone biosynthesis and this activity was highest at 24 h. CHS forms chalcone precursors for isoflavonoids and this activity was maximal at 30 h. Isoflavone aglycones were highest at 48 h. IGT converts isoflavone aglycones into glucosides and its highest activity was at 72 h. The highest concentration of isoflavone glucosides was at 96 h. Daidzein is a precursor for other classes of isoflavonoids /11/ and since the Acifluorfen-treated leaves had higher IGT activity for daidzein than for formononetin (data not shown), this may explain why treated leaves accumulated more formononetin than daidzein (Table 3).

Mesophyll cells isolated from Acifluorfen-treated leaves accumulated approximately 25% of the induced isoflavone glucosides, but that had no kaempferol glycosides and only traces of isoflavone aglycones and pterocarpans. This treatment also induced activity for PAL, CHS and IGT in the mesophyll cells. Since we do not know where within the leaf most of the activity for these enzymes was located, we ascribe less significance to percentage distribution of enzyme activity in the mesophyll than to flavonoid induction in a tissue in which these compounds are normally absent.

CONCLUSIONS

All of these results indicate a high degree of tissue compartmentalization of flavonoid biosynthesis and accumulation within the leaf. They further indicate that: 1) flavonoids may be distributed between leaf tissues of one species in a particular pattern, but they may be found in a completely different group of tissues in the leaves of another species, 2) flavonoid biosynthesis can be induced in tissues which do not normally accumulate flavonoids, and it seems clear that 3) studies on the subcellular localization of flavonoid biosynthesis in differentiated plant parts should be coupled with tissue localization studies. If, for example, flavonoids accumulate only in the epidermis, little insight will be gained by careful fractionation and analysis of mesophyll cells.

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CHLOROPLASTS OF HIGHER PLANTS ARE THE SITE OF SYNTHESIS OF PRIMARY AROMATIC COMPOUNDS: COMPARTMENTATION AND PARTIAL CHARACTERIZATION OF CRUCIAL ENZYMES OF THE SHIKIMATE PATHWAY

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ABSTRACT

Recent studies on the synthesis of aromatic amino acids revealed that chloroplasts are the main site of the shikimate pathway. Most of enzymes were identified and some were isolated from the soluble chloroplast protein fraction (chloroplast stroma): DAHP-synthase, DHQ-synthase, SORase/ DHQase and SK. The pathway is regulated by a feedback in a mode specific for chloroplasts.

The enzymes of cinnamic acid synthesis were only found in the cytosol. Therefore the secondary metabolism appears to be segregated from the primary one by its localization in the cytosol.

On the other hand,the plastidic prenylquinones originating from homogentisate, namely plastoquinone as an essential compound in photosynthetic electron transport and tocopherol as the biological scavenger for energized species of oxygen, are formed solely at the inner membrane of the chloroplast envelope. Phylloquinone, a minor compound in higher plants, is also formed at chloroplast membranes.

ABBREVIATIONS

DAHP-synthase, Deoxy-D-arabinoheptulosonate-7-phosphate-synthase, EC 4.1.2.15; DHQ-synthase, dehydroquinate-synthase, EC 4.6.1.3; SORase/DHQase, shikimate oxidoreductase/dehydroquinate hydrolyase, EC 1.1.1.25 / EC 4.2.1.10; SK, shikimate kinase, EC 2.7.1.71; EPSP-synthase, enolpyruvylshikimate 3-phosphate-synthase, EC 2.5.1.19; reversible GAPDH, reversible glyceraldehyde 3-phosphate dehydrogenase, EC 1.2.1.13; non-reversible GAPDH, non-reversible glyceraldehyde 3-phosphate dehydrogenase, EC 1.2.1.9; E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate; DTT, dithiothreitol; S3P, shikimate 3-phosphate; U, unit of enzyme activity, μmol x min^{-1};

INTRODUCTION

The aromatic precursors of the prenylquinones (tocopherols, plastoquinones, phylloquinone) are supplied by the shikimate pathway. This pathway was shown to be active in the soluble fraction of plant tissues. Its intracellular localization was not clear as yet. On the other hand, the subsequent steps of prenylquinone synthesis were doubtlessly restricted to chloroplast membrane fractions (envelope and thylakoids) /1/. Therefore it seems probable that the aromatic precursors of prenylquinones originate inside the plastids, too. Consequently our research focussed on the intracellular localization of the shikimate pathway. Experiments were made with spinach and - to a less extent - pea leaves.

The incorporation of $^{14}CO_2$ and $|^{14}C|$shikimate into tyrosine, phenylalanine and tryptophan /2, 3, 4/ as well as the incorporation of L-$|$ring-3,5-$^{3}H|$-4-hydroxyphenylpyruvate into tyrosine /5/ and the synthesis of $|^{14}C|$phylloquinone arising from $|^{14}C|$shikimate /6/ by spinach chloroplast fractions suggests that the complete shikimate pathway exists in chloroplasts.

The stroma of plastids from spinach leaves was recently found to fulfill the requirements of shikimate-turnover. A specific protein, the SORase, strictly associated with another shikimate pathway enzyme, the DHQase /7/ could be purified to apparent homogeneity /8/. The ratio of SORase/DHQase-activities (ca 9:1) remained fairly constant throughout the purification procedure. Evidence for the bifunctionality of this protein was presented in an abstract and a preceding paper /10/.

We have now used an essentially different approach to provide further evidence that crucial enzymatic activities of the shikimate pathway, i.e. SORase/DHQase and SK are restricted to the soluble chloroplast extract of spinach.

MATERIALS AND METHODS

Chemicals

$|1-^{14}C|$PEP was purchased from Amersham Buchler (Braunschweig, F.R.G.). DEAE-Sepharose CL-6B and Sephacryl S 300 were obtained from Pharmacia, hydroxylapatite Bio-Gel HPT from Bio Rad, cellulase Onozuka R 10 and macerase Maceroenzyme R 10 from Serva (Heidelberg, F.R.G.), glyphosate (N-phosphonometylglycine) from Ehrenstorfer (Augsburg, F.R.G.). DAHP was prepared according to Herrmann and Poling /11/ from E4P which was synthesized according to Sieben et al. /12/. Dehydroquinate was synthesized according to /13/. |U-^{14}C|shikimate was synthesized from $^{14}CO_2$ with the aid of glyphosate inhibition of EPSP-synthase from tomato leaves as reported by P. Gehrke and Prof. N. Amrhein (Bochum) in a personal communication. All other chemicals were of analytical grade.

Plant Material

Spinach plants (Spinacia oleracea) were greenhouse grown.

Preparation of Total Leaf Extract.

Washed deribbed leaves (100 g) were homogenized by a Waring blendor in 100 ml of buffer, either 50 mM Tris/HCl pH 7.5, 1 mM EDTA, 1.2 mM phenylmethylsulfonylfluoride, 0.4 mM DTT (buffer I) in experiments concerning SORase and DHQase; 10 mM sodiumcitrate pH 6.8, 2 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 0.4 mM E4P (buffer II) in experiments with DAHP-synthase; or 10 mM glycine/NaOH pH 10.0, 10 mM $MgCl_2$, 5 mM DTT (buffer III) in experiments with SK. Following filtration and subsequent centrifugation (105,000 g for 30 min), the supernatant was used as total leaf extract.

Isolation of Chloroplasts and Chloroplast Stroma

Intact chloroplasts were isolated as described previously /5/ and freed from other organelles and most cytosolic enzymes by centrifugation through a linear Percoll density gradient /5/. The soluble contents of the stroma were released by osmotic shock in hypotonic solution of buffer I, II or III. Plastidic membranes were centrifuged down and discarded.

Isolation of Protoplasts, Cytoplasm and Chloroplast Stroma

Protoplasts were prepared by a method modified from Edwards et al. /14/. They were purified on a discontinuous gradient and then ruptured mechanically /14/. Intact chloroplasts were completely separated from cytoplasm by repeated centrifugation (2000 g, 2 min, three times) and the stroma was obtained as described above. Marker enzymes of cytoplasmic and stroma fractions were measured to determine cross contaminations.

Separation of Chloroplast Subfractions

Envelopes, thylakoids and the soluble chloroplast fraction were prepared from purified chloroplasts by the method of Douce and Joyard /15/, except the media contained all the additions described for buffer I, II or III.

Criteria of Purification

For criteria of the purity of chloroplasts and their subfractions see /5, 15/.

Gelelectrophoresis and Staining for Enzyme Activity

Analytical disc-electrophoresis was performed with 7.5 % and 10 % acrylamide gels, pH 7.9 /16/. The stacking gels were polymerized with the aid of riboflavin. Gels were held at 4° C during electrophoresis. They were stained for SORase activity with 10 ml of a solution consisting of 100 mM glycine/NaOH buffer, pH 9.5, 0.8 mM NADP, 3 mM shikimate, 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 0.35 mM phenazine methosulfate. The SORase bands could be recognized within five min after incubation at room temperature. The gels were alternatively stained or assayed for enzyme activity.

Assay of DAHP-Synthase, DHQ-Synthase, DHQase, SORase, and SK

DAHP synthase was measured by following the incorporation of $|^{14}C|$PEP into DAHP. Standard incubation mixtures (final volume 0.1 ml) contained 0.2 M sodium citrate, pH 6.8, 0.4 mM E4P, 0.4 mM PEP including 0.2 µCi $|^{14}C|$PEP and the indicated amounts of protein extract. Mixtures were incubated at 20 ± 2° C for 20 min, then 100 µl methanol:acetone (1:1, v/v) were added to stop the reaction. Following centrifugation (2 min) to remove proteins, the supernatant was subjected to TLC on Kieselguhr-Silicagel 60 precoated plates (Merck, Darmstadt, F.R.G.), together with 20 µl DAHP (0.6 mM) as carrier. The chromatogram was developed using ethanol:ammonium acetate (1 M, pH 5.0) (7:1, v/v). TLC plates were checked with a radioscanner (Berthold Silena) for accurate separations. The R_f-values were: DAHP 0.72, PEP 0.21, pyruvate (from slight decomposition of PEP) 0.84. Following spraying with 0.05 % Rhodamine B - ethanol (w/v) the visualized DAHP-zones were scraped out and counted in a liquid scintillation counter (Packard Tricarb 3255). Control assays were done in the same way except that the mixture was stopped immediately after addition of samples. Prior to the assay, the DAHP-synthase was separated from phosphatase activities by ion-exchange chromatography. Cellular fractions containing DAHP-synthase were loaded onto a column of DEAE-Sepharose equilibrated with buffer II and washed with this buffer until the absorbance (at 280 nm) of the effluent was negligible. Then the enzyme was eluted with a linear gradient of 0 to 0.5 M KCl in the same buffer. The active fractions (70 to 90 mM KCl) were pooled and used for assays and further experiments.

Fig. 1 clearly shows the direct conversion of $|^{14}C|$PEP into $|^{14}C|$DAHP without interfering activities (e.g. phosphatases).

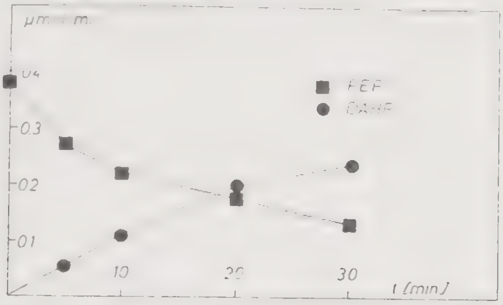

Fig. 1. Time Course of the Conversion of $|^{14}C|$PEP into $|^{14}C|$DAHP.
Rates were determined by scraping out and counting both, DAHP and PEP. The assay was performed as described in materials and methods with DAHP-synthase from DEAE Sepharose chromatography.

DHQ-synthase was assayed by measuring the decrease of DAHP (0.4 mM DAHP, Tris/HCl buffer, pH 7.5), essentially according to /17/.
DHQase was assayed spectrophotometrically by measuring the formation of 3-dehydroshikimate at 234 nm. The assay mixture contained 0.1 M Tris/ HCl, pH 8.0, and 0.2 mM 3-dehydroquinate in a final volume of 0.5 ml.
SORase was routinely assayed in the reverse direction by measuring the increase in absorbance at 340 nm during the oxidation of shikimate to 3-dehydroshikimate (NADPH-formation) in 0.1 M glycine/NaOH buffer, pH 9.5, 2 mM shikimate, 0.5 mM NADP.
SK was measured by following the incorporation of $|^{14}C|$shikimate into S3P, essentially according to /18/ but modified as follows. The incubation mixtures (final volume 0.1 ml) contained 50 mM glycine/NaOH, pH 10.0, 5 mM $MgCl_2$, 5mM DTT, 4 mM ATP, 0.1 to 1.0 mM shikimate including 0.02 to 0.2 µCi $|^{14}C|$shikimate and the indicated amounts of protein extract. After incubation at 30° C for 10 to 80 min the reaction was stopped by the addition of 25 µl of 8.8 % (w/v) trichloracetic acid. Following centrifugation to remove proteins, the supernatant was subjected to an Eppendorf tip filled with 200 mg of Dowex 1x8, Cl^-. Elution of shikimate was obtained with 250 µl 0.3 M acetic acid (6 times). S3P was eluted in a similar way except 1 M NaCl was used. After addition of 12 ml of Hydroluma (Baker) the samples were counted in a liquid scintillation counter (Packard Tricarb 3255).

Assay of Non-reversible GAPDH and Reversible GAPDH

Determination of cross contaminations of protoplast fractions was done by measuring both the non-reversible GAPDH, a marker enzyme of cytoplasm (assay according to Kelly and Gibbs /19/), and a marker enzyme of stroma, the reversible GAPDH (assay according to Stitt et al. /20/).

Other Methods

CO_2 fixation rate, chlorophyll as well as protein contents were determined as described in /5/.

RESULTS

Assay of DAHP-Synthase, DHQase, SORase and SK in Chloroplast and Cytoplasmic Fractions Obtained from Protoplasts

Protoplast fractionation techniques were used to elucidate the problem of the distribution of shikimate pathway enzymes within spinach parenchym cells. The fractionation technique as in /14/ was applied which allows separation of plastidic and cytoplasmic fractions from protoplasts (Tables 1 and 2).

Table I Distribution of Shikimate Pathway Enzymes and Marker Enzymes in Protoplast Subfractions.

The shikimate pathway enzymes SORase, DHQase and DAHP-synthase as well as marker-enzymes for the chloroplast stroma and the cytoplasm were assayed as described in materials and methods.

	Enzyme									
	DHQase		SORase		DAHP-synthase		rev. GAPDH		non-rev. GAPDH	
Fraction	U/Fraction	%	U/Fraction	%	U/Fraction	%	U/Fraction	%	U/Fraction	%
Protoplasm	0.0180	100	0.232	100	0.0088	100	2.12	100	0.367	100
Stroma	0.0156	87	0.205	88	0.0019	22	1.89	89	0.0037	1
Cytoplasm	0.0024	13	0.027	12	0.0069	78	0.23	11	0.363	99

The protein concentrations were 14.0 mg/ml stroma (fraction volume 0.6 ml) and 4.0 mg/ml cytoplasm (fraction volume 2.04 ml).

Table 2 Distribution of Shikimate Pathway Enzymes and Marker Enzymes in Protoplast Subfractions.

The shikimate pathway enzymes SK and SORase as well as marker-enzymes for the chloroplast stroma and the cytoplasm were assayed as described in materials and methods.

	Enzyme							
	SK		SORase		rev. GAPDH		non-rev. GAPDH	
Fraction	U/Fraction	%	U/Fraction	%	U/Fraction	%	U/Fraction	%
Protoplasm	0.0432	100	0.115	100	3.108	100	0.112	100
Stroma	0.0419	97	0.097	85	2.661	86	0.006	5
Cytoplasm	0.0013	3	0.017	15	0.447	14	0.106	95

The fraction volumes were 0.7 ml stroma and 1.6 ml cytoplasm.

The distribution of marker enzymes (Tables1 and 2) reveals that the stroma is virtually free of cytoplasmic enzymes, though the cytoplasmic fraction is considerably contaminated by chloroplast enzymes. Comparing the distribution of marker enzymes verifies that the activities of SORase, DHQase and SK are confined to the fraction of soluble chloroplast protein (stroma) since activity was not detected in the cytoplasm. DAHP-synthase is active in both fractions.

Assay of DAHP-Synthase, DHQase, SORase and SK in Chloroplast Subfractions

Activity of DAHP-Synthase, DHQase and SORase could be detected only in the chloroplast stroma fraction, but not in the chloroplast membrane fractions (thylakoids and envelopes). The main activity of SK was also found in the stroma fraction. However, some activity was detectable in the thylakoid fraction, which could be eluted by few washings.

Comments upon DAHP-Synthase Assay

The colorimetric thiobarbituric acid assay of DAHP-synthase, essentially according to Warren /17/, turned out to be unsuitable for our purposes. A number of reagents interfered with the assay method including stabilizing agents for the enzyme. This becomes significant as the enzymatic activity is low in higher plants and especially with preparations of plastids and their subfractions. In the presence of protecting reagents (reagents and their concentrations are given in brackets) the sensitivity of the colorimetric assay (measured spectrophotometrically at a wavelength of 549 nm) decreased to 70 % (1 mM DTT), 75 % (1 mM 2-mercaptoethanol), 38 % (1 mM $MnCl_2$) and 80 % (0.5 % PVP (w/v)), respectively.

The possibility of artifacts, like those listed above, could be minimized using the radioactive assay introduced here. Additionally, the sensitivity of this improved method was 8 to 10 times higher as compared to the conventional assay (ca. 1 pmol DAHP min^{-1} ml^{-1} could be detected).

Indications to DAHP-Synthase Isoenzymes

At present the striking distribution of DAHP-synthase activity in chloroplast and cytosolic fractions - different from that of DHQase, SORase and SK - is the unique unequivocal indication for two isomers of DAHP-synthases. Both isozymes are stabilized by E4P, Mn^{2+} or Mg^{2+} and 2-mercaptoethanol as additions in buffer II. Sodium citrate, pH 6.8 proved to be the most suitable buffer tested. The cytosolic enzyme is activated (2-fold), chloroplast isozyme not at all, by phenylalanine (1mM). Both DAHP-synthases have extremely high affinities to E4P (K_m ca. 0.7 uM) and a K_m in the range of 0.3 mM for PEP. They are strongly inhibited by higher salt concentrations: $(NH_4)_2SO_4$, $MgSO_4$ and KCl at concentrations of 0.8 M and 1.6 M decrease the DAHP-synthase activity to 20 % and 10 %, respectively. NH_4Cl showed less inhibition; at 0.8 M and 1.6 M the remaining DAHP-synthase activities were 46 % and 38 %.

Neither the plastidic nor the cytoplasmic isozyme could be inhibited significantly by glyphosate (concentrations up to 1 mM).

Localization of DHQ-Synthase in the Chloroplast Stroma

Although the second enzyme of the shikimate pathway, DHQ-synthase, has been detected in the stroma fraction of spinach chloroplasts (data not shown), it is not clear at present whether or not there is any activity in the cytoplasm.

Chromatographic and Electrophoretic (PAGE) Procedures for Separation of Potential Isozymes of SORase/DHQase

The bifunctional protein (SORase/DHQase) was always found in the same fractions of a DEAE-Sepharose chromatography regardless whether applying stroma or total leaf extract.

Eluting the DEAE-Sepharose column with salt gradients up to 1 M KCl yielded identical elution profiles /10/. Neither gelfiltration on Sephacryl S 300 nor hydroxylapatite chromatography were able to detect an additional peak of SORase or DHQase activity deriving from cytoplasmic isozymes.

All assays were repeated with different buffers and several pH-values (sodium citrate, pH 6.8; Tris/HCl, pH 7.0; Tris/HCl, pH 8.0; glycine/NaOH, pH 9.5) to exclude the possibility that the presence of isozymes was missed only because of the possible different pH-optima or salt sensitivity.

When soluble protein fractions obtained from purified chloroplasts or total leaf homogenate were subjected to non-denaturing PAGE and specifically stained on the gel for enzyme activity, no band of an extraplastidic SORase was detectable. However, both gels showed a characteristic staining pattern. Four bands with enzyme activity were recognized. In parallel experiments gel slices were obtained from the same PAGE as used in SORase activity determination. They were cut out at the identical R_f-values as for SORase activity and eluted in buffer. The DHQase assay revealed that only gel slices known to contain SORase activity showed DHQase activity. This may be interpreted as a group of four distinct enzymes (all inside the chloroplast) with such a similarity that only PAGE is able to separate them. For a more detailed discussion concerning these multiple forms see the preceding paper /10/.

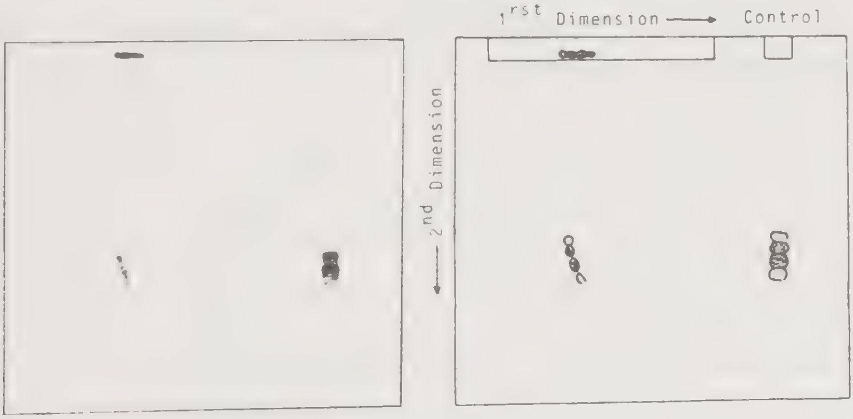

Fig. 2. Two-dimensional PAGE of SORase/DHQase.
The multiple forms of SORase/DHQase were separated on a rod gel (7.5 % polyacrylamide, 0.005U SORase per gel) and then subjected to further electrophoresis in the second dimension on a slab gel with the same polyacrylamide concentration. Gels were run and specifically stained for SORase activity. A gel of simultaneously run enzyme is depicted at the top of the figure, an additional sample was run only in the second dimension. All multibanded patterns are essentially the same, clearly proving that isozymic configurations were not affected by electrophoresis. The additional line drawing facilitates to distinguish the gel patterns.

Two-dimensional PAGE ruled out the possibility that these forms were due to an artifact during electrophoresis. The multiple forms of SORase/ DHQ-ase were separated on a rod gel and then subjected to further electrophoresis in the second dimension on a slab gel. Gels were specifically stained for SORase activity. An additional sample was run only in the second dimension showing no change between the distinct forms. There was no convertibility between the individual bands proving that the isozymic configurations were not attacked by the electrophoretic procedure (Fig. 2).

Properties of SK

As described above, SK could be detected mainly in the chloroplast stroma fraction. Our experiments starting from spinach protoplasts clearly exclude the existence of a cytoplasmic isozyme in a relevant proportion. Because of the contamination of the cytoplasmic fractions with proteins from chloroplast stroma we made a further approach to prove that the SK activity detected in cytoplasm originates from the stroma. Comparing the pH-optima of SK in chloroplast stroma and cytosolic fractions (Fig. 3) shows that the enzyme behaves in both fractions like a chloroplast stroma protein exhibiting its maximal activity in an alkaline medium. Thus, the activity measured in cytosolic fractions belongs to contamination from broken chloroplasts (for the distribution of the stroma enzyme, compare Table 2).

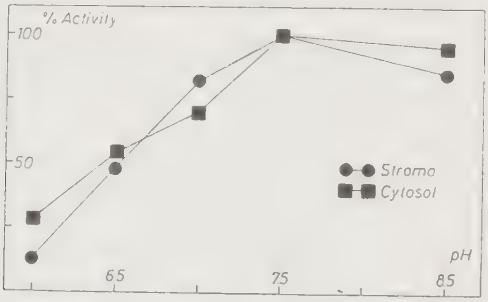

Fig. 3. pH-Dependence of the SK Activities Found in Chloroplast Stroma and Cytoplasmic Fraction Prepared from Spinach Protoplasts.
Assays were performed with 100 mM Bistrispropane including 10 mM NaF and 1 mM shikimate. The SK activities were 0.0764 U x ml^{-1} for the stroma fraction (100 %) and 0.00063 U x ml^{-1} for the cytoplasmic fraction (100 %).

A precise determination of the pH-optimum of SK was done with different buffers (Fig. 4), essentially as described in Materials and Methods.

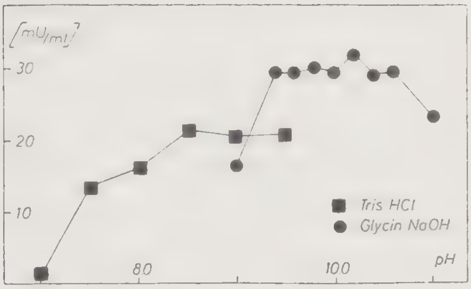

Fig. 4. Dependence of SK Activity on pH.
Concentrations were as follows: buffer 50 mM, NaF 20 mM, shikimate 0.2 mM.

As expected, activity of SK was strongly Mg^{2+}-dependent. There was no activity without Mg^{2+}. Detailed information is depicted in Fig. 5.

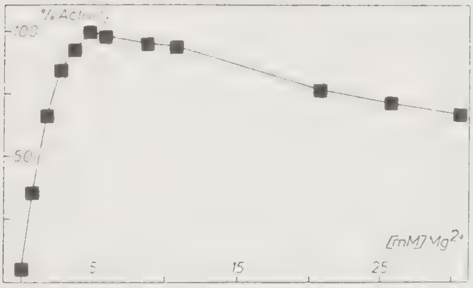

Fig. 5. Effect of Mg^{2+} on the Activity of SK.
Assays were incubated as described above. 100 % activity corresponds to 0.0396 U x ml^{-1}. The metal ion concentration was varied from 0 to 31 mM.

The determination of the Michaelis constant of SK for shikimate resulted in a value of 0.25 mM.

DISCUSSION

Localization of Shikimate Pathway in Chloroplasts

The data presented in this paper demonstrate the localization of SORase/DHQase and SK in the soluble chloroplast extract. The specific distribution of other enzymes of the shikimate pathway between the chloroplast and the surrounding cytoplasm in spinach leaf cells is not as clear yet. At the present stage of our research results here show that DAHP-synthase exists in duplicate forms, one located inside the chloroplast, the other in the cytoplasm, suggesting it has a function other than biosynthesis of aromatic amino acids. DHQ-synthase has at least been found in the chloroplast stroma fraction (data not shown).

The following reactions, catalyzed by SORase/DHQase are confined to the chloroplast stroma. The same appears to be the case for EPSP-synthase. O. Joop and Prof. N. Amrhein (Bochum) reported in a personal communication that there were no signs for extraplastidic isozymes of EPSP-synthase in spinach leaves (for isolation of EPSP-synthase see /22/). These data clearly align the shikimate pathway with the chloroplast as a prerequisite of a primary aromatic metabolism in plastids (see below).

Evidence for an Incomplete Pathway in the Cytosol

There also appears to be an additional, non-complete shikimate pathway in the cytoplasm of spinach. Our studies verify the existence of a cytosolic DAHP-synthase. Maybe this enzyme is necessary to supply quinic acid, starting from glucose outside the plastids /23/.The occurrence of some enzymes of the shikimate pathway in both compartments could be an interesting point in terms of regulation. Especially the problems in turnover of quinic acid which accumulates in many plants /23/ is an ambiguous subject which requires more detailed studies. A particular review from A. Boudet will deal with this topic (personal communication Prof. A. Boudet, Toulouse).

The exclusive localization of the shikimate pathway in chloroplasts was proved only for spinach leaves. However, it is possible that in other species, especially those owning a marked secondary metabolism (e.g. corn /24/ or oak seedlings /23/) a second shikimate pathway is present outside the chloroplasts. Indeed, some evidence for this hypothesis was provided by recent studies /25, 26/.

Control of Coexisting Metabolic Pathways

The Calvin cycle and the shikimate pathway are housed in the same compartment, the chloroplast stroma. The fact that both biosynthetic routes compete for E4P could cause problems. We think these questions could be solved by considering the different kinetic properties of DAHP-synthase, sedoheptulose 1,7-diphosphate aldolase and transketolase.

DAHP-synthase has a very high affinity for E4P (ca 0.7 µM), but the velocity of its turnover is very low (0.2 nmol x min^{-1}x mg^{-1} protein from total leaf extract). On the other hand, the corresponding values of transketolase are vice versa (K_m in the range of 100 to 130 µM, the specific activity is about 4 to 40 nmol x min^{-1}x mg^{-1} protein, respectively (data from /27, 28/). Unfortunately, the values for sedoheptulose 1,7-diphosphate aldolase from spinach have not yet been accurately determined. Nevertheless, the following statement is conceivable. Whenever E4P concentration is high, there is a rapid turnover via the Calvin cycle but E4P concentration does not drop under a certain level. Because of the much higher affinity of DAHP-synthase for E4P, the anabolic flow through this pathway parallel to the Calvin cycle is not diminished.

The Biosynthesis of Primary Aromatic Compounds in the Chloroplasts: a Survey

As published earlier /2/, $^{14}CO_2$ or labelled shikimate is incorporated into aromatic amino acids and to a low but marked extent also into the prenylquinones plastoquinone-9 and α-tocopherol in intact purified spinach chloroplasts (Fig. 6). Both the aromatic amino acid and the prenylquinone synthesis are regulated by an endproduct feedback of the shikimate pathway /29/. Higher amounts of phenylalanine or tyrosine inhibit their own synthesis, however, only tryptophan lowers the synthesis of all three amino acids and therefore centrally affects the shikimate pathway at a stage between shikimate and chorismate. Since EPSP-synthase /22/ and SK failed to be inhibited by aromatic amino acids it should be tested if chorismate-synthase would be regulated by aromatic amino acids.

The supply with substrates from the carbon metabolism to cover the requirements of the shikimate pathway is as yet open in some details. As mentioned above, E4P is supplied from the Calvin-Cycle. Recently it was shown that the concentration of PEP amounted to about 40 µM in the chloroplast /30/ and PEP is also shuttled across the chloroplast envelope membrane by the phosphate translocator /31/. At present it is under investigation whether this is the only effective system for the supply with PEP.

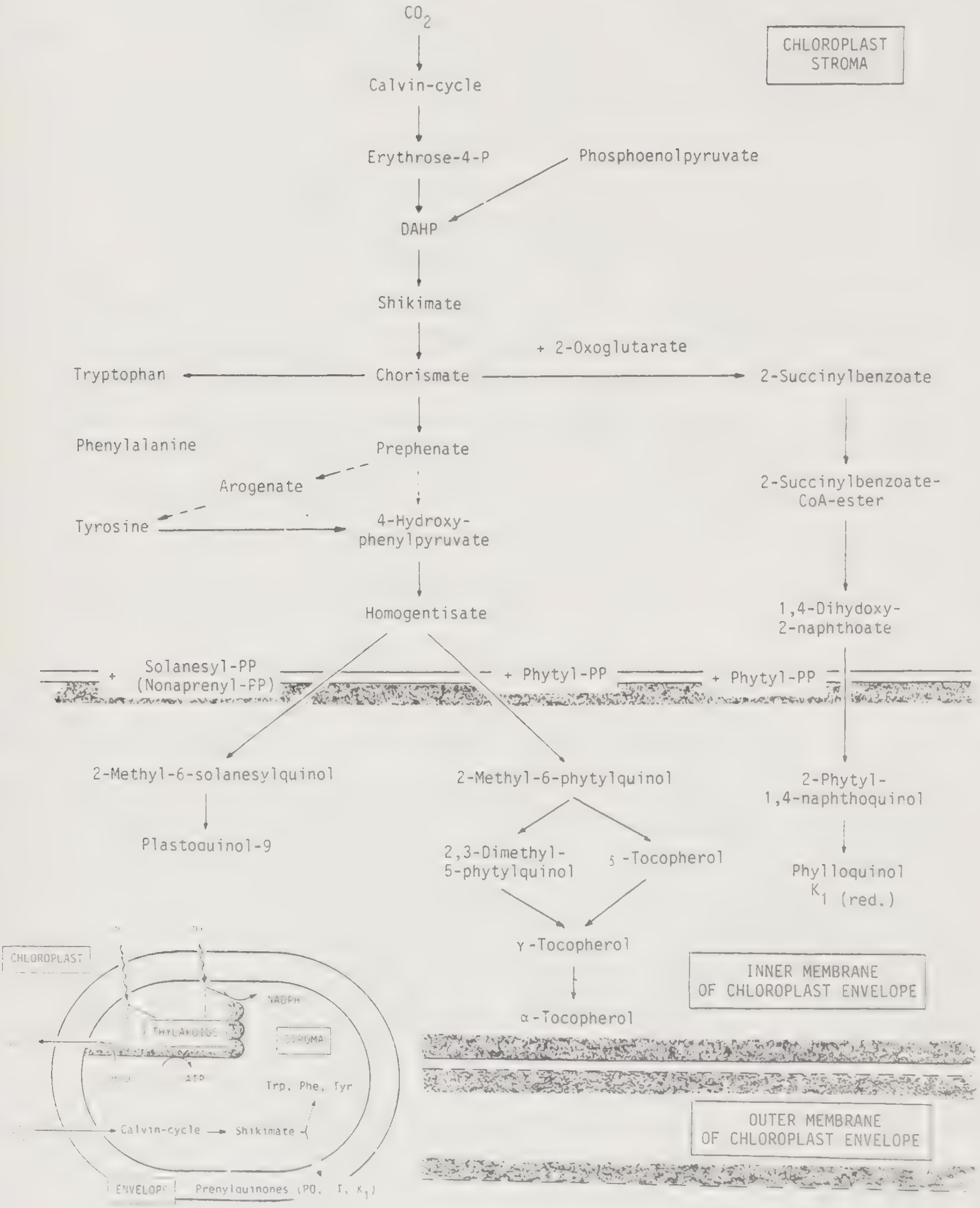

Fig. 6. Compartmentation of the Shikimate Pathway and the Prenylquinone Synthesis in the Chloroplast.

4-Hydroxyphenylpyruvate is delivered from the prephenate dehydrogenase reaction or more probably from the oxidation of tyrosine by an L-amino acid oxidase of the chloroplast thylakoid membrane /32/ or stroma in the formation of the prenylquinones α-tocopherol and plastoquinone-9. 4-Hydroxyphenylpyruvate is then oxidized to homogentisate by the 4-hydroxyphenylpyruvate dioxygenase which occurs at the stromal face of the chloroplast envelope /5/ (Fig. 7).

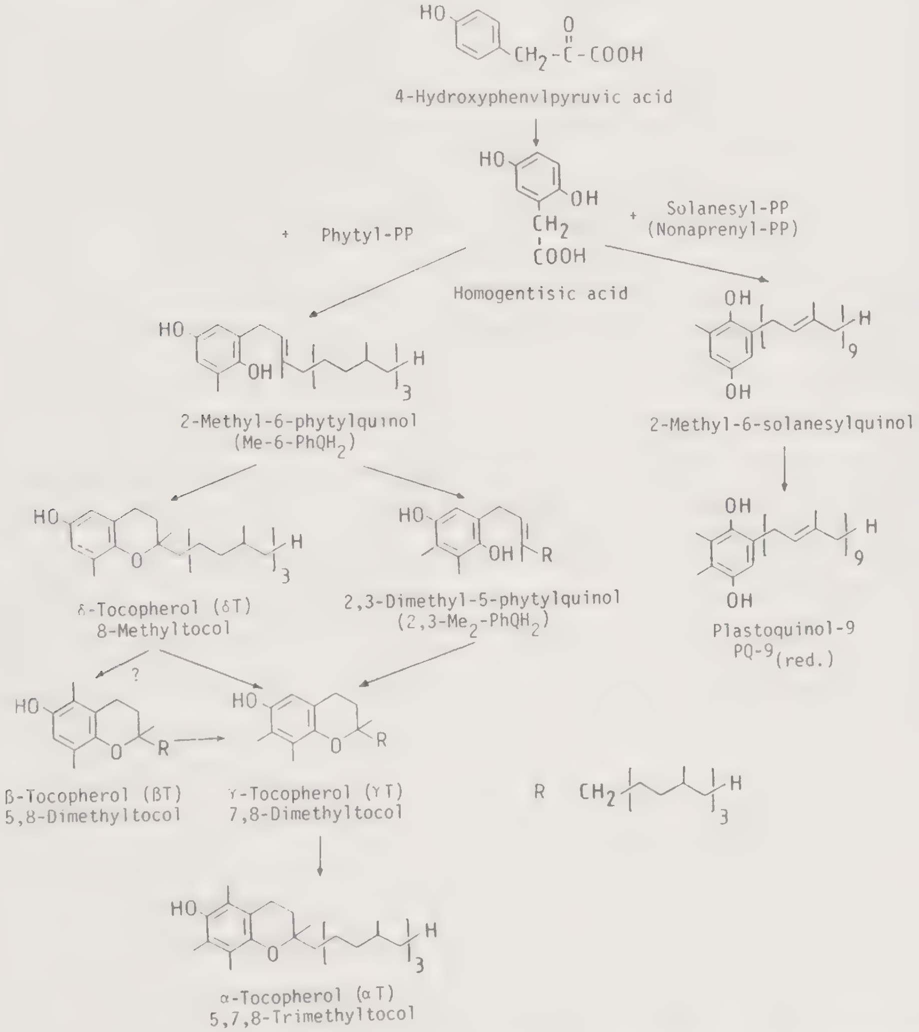

Fig. 7. Biosynthesis of α-Tocopherol and Plastoquinone-9 in Chloroplasts.

The following steps in the formation of prenylquinones are localized at the inner membrane of chloroplast envelope /33/; no enzyme activity was found in the chloroplast stroma. The prenylation reaction in α-tocopherol and plastoquinone-9 formation is strongly directed by an attack specifically on position 2 of homogentisate /34, 35/; no other position isomer occurs. The prenyl side chain always exists in the trans configuration /35/.

In α-tocopherol formation 2-methyl-6-phytylquinol is formed from homogentisate and phytyl-PP. In the following step two reactions appear to compete for the quinol as a substrate: the methylation reaction with S-adenosylmethionine to yield 2,3-dimethyl-5-phytylquinol and the cyclization reaction to yield the chromanol δ-tocopherol /36/.

2,3-Dimethyl-5-phytylquinol and also δ-tocopherol /37/ react to γ-tocopherol. It maybe that ß-tocopherol of gramineae is also formed from δ-tocopherol /38/. In spinach γ-tocopherol and also ß-tocopherol is methylated with S-adenosylmethionine to yield α-tocopherol /39/.

In the formation of plastoquinone-9 from homogentisate, the intermediate products are 2-methyl-6-solanesylquinol and plastoquinol-9. Apart from identical reaction mechanisms it appears that different enzymes are involved in the formation of α-tocopherol and plastoquinone-9. This may be supported by the fact that plastoquinone-9 synthesis occurs both at the envelope and also at the thylakoid membranes, whereas α-tocopherol synthesis is soleley restricted to the chloroplast envelope /40/.
The prenyl-PP's required in the prenylquinone formation are delivered by a plastidic mevalonate pathway in the chloroplast stroma /41, 42/ and a subsequent polyprenyl-PP synthesis at the chloroplast membranes. Phytyl-PP is formed from geranylgeranyl-PP at the chloroplast envelope by a NADPH consuming reaction /43/.

Phylloquinone (vitamine K_1) represents a minor constituent generally occurring in chloroplasts (about 1 mol per 100 moles chlorophyll in photosystem I /44/). Its role in the chloroplast metabolism is not yet known. In gram-negative bacteria menaquinones (vitamine K_2) replace ubiquinone in the electron transfer under anaerobic conditions /45/. The biosynthesis of phylloquinone in plants /46/ (Fig. 8) appears to be essentially identical to menaquinones studied extensively in bacteria /47/. It is assumed that the phylloquinone synthesis arises from the chorismate step (see also considerations in Haslam /48/); 2-succinylbenzoate (= 4-(2'carboxyphenyl) 4-oxobutyrate) is formed by introduction of the succinylsemialdehyde moiety from a thiamine-PP dependent 2-oxoglutarate decarboxylation reaction.

The benzoate formed is activated to the CoA ester which is cyclisized to 1,4-dihydroxy-2-naphthoate. The steps from the benzoate to the naphthoate were studied by Leistner's group in plant cell cultures of Galium mollugo /49, 50/. As shown by us /6/, the prenylation of the naphthoate occurs at the chloroplast envelope. Phytyl-PP is preferred to geranylgeranyl-PP and farnesyl-PP. 2-Phytyl-1,4-naphthoquinol formed is methylated in chloroplast thylakoid fractions to yield phylloquinol /51/.

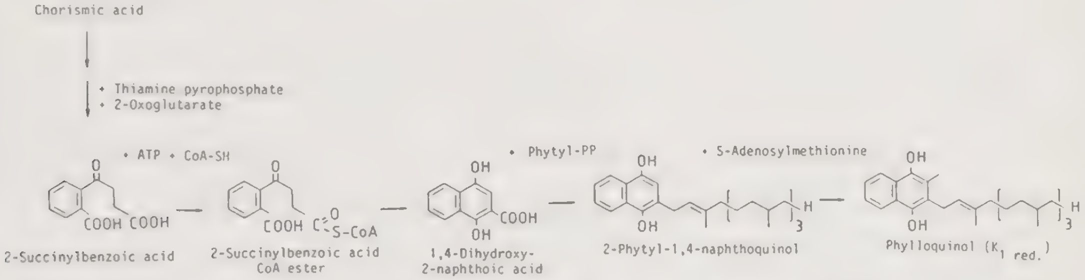

Fig. 8. Biosynthesis of Phylloquinone (Vitamine K_1) in Plants.

From the data shown it can be concluded that chloroplasts are autonomic in aromatic amino acid and prenylquinone synthesis. Moreover, experiments with organelle fractions showed a strong compartmentation inside the chloroplast. The intraplastidic localization of aromatic and prenylquinone synthesis reflects a direct relationship between the hydrophilic and lipophilic character of the substrates.

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EXTERNAL ACCUMULATION OF FLAVONOID AGLYCONES IN *ACHILLEA*

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INTRODUCTION

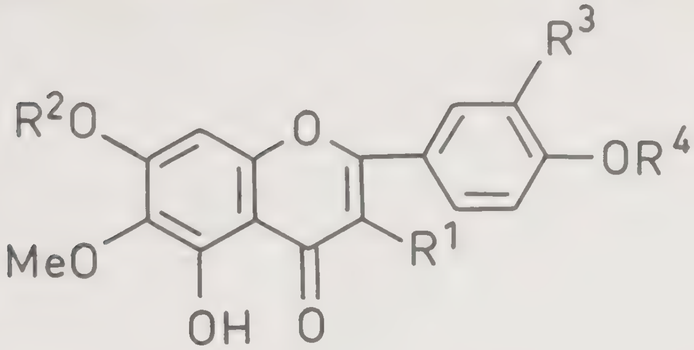
Free flavonoid aglycones have been reported to occur in various plant genera /1/. As most studies are primarily concerned with structure elucidation or distribution, the ecological importance of different accumulation sites is often neglected. However, it appears to be of significance, as flavonoid aglycones have been observed to accumulate externally as well as internally in several taxa. Correlations between external flavonoid formation and anatomical differentiation (secretory systems), as well as with ecological factors (habitats),are apparent /2/ and may explain the higher frequency of this phenomenon in groups like the Compositae /1/. Of this family, the genus Achillea had earlier been investigated regarding the systematic significance of the leaf flavonoid complement, which is composed of C-glycosylflavones, flavonol 3-O-glycosides and flavone 7-O-glycosides /3/. In addition, free aglycones were frequently observed, but not yet structurally characterized. The present reinvestigation of Achillea has revealed that species forming free aglycones accumulate them externally. Besides the detection of individual compounds and their distribution within the genus, the possible ecological and systematic significances will also need further consideration.

RESULTS AND DISCUSSION

The genus *Achillea*, comprising more than 100 species, is distributed in the Northern Hemisphere with several centers of development in semiarid and alpine regions of Eurasia /4/. Several species both of different taxonomic groups and of different geographic origin were analyzed for their potential to form external aglycones. The flavonoid fractions were obtained by rinsing vegetative parts of flowering plants with chloroform or acetone. Comparative TLC and UV-spectral data indicated the presence of 6-hydroxyflavones and -flavonols, frequently carrying several methoxy groups. The main components identified so far are listed in Tab. 1.

The substitution pattern of *Achillea* aglycones is similar to that reported previously for other *Compositae* genera /5/. Flavones such as cirsimaritin (**2**), salvigenin (**4**), eupatorin (**6**) and 6-hydroxyluteolin 6,7,3',4'-tetramethyl ether (**7**) were frequently found in exudates (for formulae see Tab. 1). Flavone **7** has already been isolated together with quercetin 3,3',4'-trimethyl ether from *A. santolina* collected in Egypt /6/; the latter compound, however, has not been detected during the present survey. The flavonols present in many *Achillea* species (methylbetuletol **10**, 6-hydroxykaempferol 3,6,7,4'-tetramethyl ether **11**, centaureidin **13**, casticin **14**, artemetin **15**) showed a substitution pattern similar to that of the flavones. Monomethyl ethers of flavones (hispidulin **1**, nepetin **5**) and dimethyl ethers of flavones (pectolinarigenin **3**) and of flavonols (6-hydroxykaempferol 3,6-dimethyl ether **8**) appeared to be less abundant. Generally, the substitution pattern of the main flavones and flavonols showed a tendency to 6- and 4'-O-methylation. Comparably, co-occurring flavonol 3-O-glycosides including 6-O-methylated derivatives or C-glycosylflavones were exclusively 3'-O-methylated /3/, which might have some significance for considerations about biosynthetic pathways.

Table 1: Flavonoid aglycones found in *Achillea*



		R_1	R_2	R_3	R_4
1	Scutellarein 6-Me (hispidulin)	H	H	H	H
2	" 6,7-diMe (cirsimaritin)	H	Me	H	H
3	" 6,4'-diMe (pectolinarigenin)	H	H	H	Me
4	" 6,7,4'-triMe (salvigenin)	H	Me	H	Me
5	6-Hydroxyluteolin 6-Me (nepetin)	H	H	OH	H
6	" 6,7,4'-triMe (eupatorin)	H	Me	OH	Me
7	" 6,7,3',4'-tetraMe	H	Me	OMe	Me
8	6-Hydroxykaempferol 3,6-diMe	OMe	H	H	H
9	" 3,6,7-triMe (penduletin)	OMe	Me	H	H
10	" 3,6,4'-triMe (methylbetuletol)	OMe	H	H	Me
11	" 3,6,7,4'-tetraMe	OMe	Me	H	Me
12	Quercetagetin 3,6,7-triMe (chrysosplenol)	OMe	Me	H	H
13	" 3,6,4'-triMe (centaureidin)	OMe	H	OH	Me
14	" 3,6,7,4'-tetraMe (casticin)	OMe	Me	OH	Me
15	" 3,6,7,3',4'-pentaMe (artemetin)	OMe	Me	OMe	Me

With respect to taxonomic and ecological differences, a few species of *Achillea* have been selected to illustrate various accumulation trends (Table 1). In particular, the xerophytic members of *Achillea* sect. *Santolinoidea* DC.were found to accumulate aglycones externally, as has already been demonstrated for several other *Compositae* plants occurring in semiarid regions /2/. The morphology of these *Achillea* species reflects their adaptation to the xeric habitat, both in typical growth forms and the reduced leaf structures. Their area of distribution is within the Eastern Mediterranean Region /3/. Their aglycone pattern consisted predominantly of flavones and a few flavonol derivatives in addition. A typical member of the *Santolinoideae* is *A. cretica* L., occurring on some Aegean Islands (e.g. Crete) /3/. One collection of this taxon accumulated cirsimaritin (2), pectolinarigenin (3) and 6-hydroxyluteolin 6,7,3',4'-tetraMe (7) together with artemetin (15). Some of these compounds (2,7,15) were also detected in the exudate of a wild population of *A. spinulifolia* Fenzl ex Boiss.; additionally, the flavones salvigenin (4), eupatorin (6), traces of nepetin (5) and of the flavonol chrysosplenol (12) as well as minor amounts of the 6-hydroxykaempferol 3,6,7,4'-tetraMe (11) were accumulated. *A. spinulifolia* is distributed locally in S. Turkey, and, on the basis of morphological characters (e.g. white-ivory ligules), is probably more closely related to *A. cretica* /3/. Another group of the *Santolinoideae* is characterized by yellow ligules and includes species such as *A. falcata* L. and *A. lycaonica* Boiss. & Heldr., both distributed in different regions of Turkey /3/. Their aglycone profile, however, resembled largely that of the other two taxa, with the flavones 2,4,7 and the flavonol 15 as the main components. Although data on infraspecific variability have not yet been obtained, the profiles observed so far suggest a relative uniformity within this section. Since the C-glycosylflavones accumulated in the leaves of these species exhibited a similar uniformity as well /3/, both internal and external accumulation trends might be of ecological significance.

Table 2: Flavonoids found in some Achillea species

ACHILLEA SPECIES	FLAVONOID AGLYCONES (formulae, see Tab. 1)															HABITAT
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
sect. Santolinoidea																
A. cretica		○	●				●								●	xeric
A. spinulifolia		●		●	t	●	●				○	t			●	xeric
A. falcata		○		●		○	●							t	●	xeric
A. lycaonica		○	t	●			●				●				●	xeric
sect. Ptarmica																
A. abrotanoides										●	t		●	t	●	alpine, calcicole
A. moschata								●		●			●			alpine, calcifuge
A. ageratifolia																alpine, calcicole
subsp. ageratifolia									●			○				
subsp. serbica	○				t			●								
subsp. aizoon	○				○											
sect. Millefolium																
A. nobilis											●			●	●	xeric
A. filipendulina													●			xeric

● great amounts ○ small amounts t traces

Apart from xerophytes, the genus *Achillea* comprises also a series of alpine taxa, grouped within sect. *Ptarmica* (Mill.) Koch. Large, often solitary capitula and entire or divided leaves characterize these species. In contrast to the scant reports on external flavonoid formation of alpine plants /7/, many alpine *Achillea* species gave positive results. However, only aromatic species produced external aglycones, thus pointing to a correlation with the presence of secretory structures (cf. 1, 2). The main compounds accumulated were flavonol derivatives, whereas the corresponding flavones appeared to be of limited distribution. One geographic collection of *A. abrotanoides* Vis., a taxon occurring on calcareous rocks along the S. Balcanic Peninsula /4/, was found to accumulate methylbetuletol (10), centaureidin (13), artemetin (15) and traces of 6-hydroxykaempferol 3,6,7,4'-tetraMe (11) and casticin (14). A less diversified pattern, consisting of 6-hydroxykaempferol 3,6,-dimethyl ether (8), methylbetuletol (10) and centaureidin (13) was typical of *A. moschata* Wulf. This species is distributed mainly in Central Europe and prefers non-calcareous habitats /4/. Thus, the formation of external aglycones is correlated rather with the alpine habitat than with the chemistry of the substrate.

Within the S-balcanic calcicole *A. ageratifolia* (Sibth. & Smith) Boiss. aggregate, taxon delimitation appears to be problematic (R. Franzén, pers. comm.). This complex taxonomic situation is reflected to a certain extent by the incoherent aglycone composition of the three recognized subspecies. Whereas subsp. *ageratifolia* exhibited a flavonol dominated profile (penduletin 9, chrysosplenol 12), the flavones hispidulin (1) and nepetin (5) together with 6-hydroxykaempferol 3,6-dimethyl ether (8) were accumulated in subsp. *serbica*. One collection, which was morphologically close to subsp. *aizoon*, showed the formation of flavones (1, 5) only. As sufficient data on the extent of infraspecific variability have not yet been obtained, systematic conclusions cannot be drawn at present.

With the exception of a few taxa, most groups of the morphologically diverse sect. *Millefolium* (Adans.) Koch accumulated free aglycones. *A. nobilis* L., a species occurring from SE-Europe to Turkey in mostly dry habitats /3/, appeared to accumulate primarily flavonols. Its aglycone profile was composed of 6-hydroxykaempferol 3,6,7,4'-tetramethyl ether (11), casticin (14) and artemetin (15) as main compounds. The same compounds have already been isolated from flower heads of *A. millefolium* L. collected in N-America /8/, probably being part of excreted material there too. Another xerophytic member of this section, the Turkish-Iranian *A. filipendulina* Lam., accumulated mainly centaureidin (13). There are indications that infraspecific variation regarding the accumulation of major compounds is rather low in both species. Also, the main trends observed in cultivated material and wild populations of *A. nobilis* proved to be identical. Variation, however, might be expected in the occurrence of minor constituents, which have not yet been identified in most taxa.

Generally, it is assumed that external flavonoids might act as an UV-screen, thus being ecologically significant /9/. This is also indicated by their occurrence on xerophytes and alpine *Achillea* species, whereas those restricted to mesic habitats were devoid of external aglycones. Their functional role, however, has to be kept in mind if this phenomenon is to be interpreted systematically. Prior to the interpretation of this rather "new" character, factors like seasonal changes or variation of the flavonoid profile during the ontogenetic process should also be taken into consideration.

EXPERIMENTAL

Air-dried vegetative parts of flowering plants were rinsed with $CHCl_3$ or with acetone to dissolve the exudate flavonoids. Thin-layer chromatography was performed on silica and on polyamide DC-11. The solvents used were A) toluene/methylethyl ketone 9 : 1 and B) toluene/dioxane/glacial acetic acid

90 : 25 : 4 for silica, C) toluene/petrol$_{100-140^{\circ}}$/methylethyl ketone/methanol 30 : 90 : 2 : 1.5, D) toluene/petrol$_{100-140^{\circ}}$/ methylethyl ketone/methanol 60 : 30 : 10 : 5, E) toluene/ dioxane/methanol 80 : 10 : 10, and F) toluene/methylethyl ketone/methanol 60 : 25 : 15 for polyamide. Plates were viewed under UV-light (366 nm) before and after spraying with "Naturstoffreagenz A". The flavonoids were identified by direct comparison with authentic markers in different solvent systems and in several cases also by comparison of their UV-spectra /9/ with literature data.

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INVESTIGATION OF THE CHEMICAL CONSTITUENTS, PARTICULARLY THE FLAVONOID COMPONENTS, OF PROPOLIS AND *POPULI GEMMA* BY THE GC/MS METHOD

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Besides synthetic medicines, natural substances of plant, animal or mineral origin have aroused considerable interest in medicinal practice.

Propolis, a natural product from plants, can also be regarded as one of the potential sources providing pharmaceutical agents in the future.

The plant origin of propolis has been studied by many researchers. Helfenberg /1/, Jaubert /2/, Mabrv /3/, Villanueva /8/, Rosch /4/, Geismann /5/, Popravko /6/, Bankova /7/, and in Hungary, Ambrózy, and Király and others have described that propolis is collected by bees from the unopened budlets of various balsam trees, primarily of Betula, Populus, Pinus, Prunus, Acacia, Aesculus hypocastane species, and of several fruit trees.

The composition of propolis is dependent on the climate, geographical environment and on plant flora, and contains flavonoids, aromatic carboxylic acids, benzoic acid derivatives, carbohydrates, fatty acids, terpenes and inorganic materials.

Data in the literature, obtained by chemical and instrumental analytical methods, refer to a close relationship of the chemical components of propolis with those of Populi gemma.

To furnish additional evidence for the common origin of propolis and Populi gemma, comparative studies under equal conditions have been accomplished.

Both substances were extracted with various methanol-water mixtures and the extracts were fractionated with organic solvents of different polarity. The obtained fractions were subjected to further separation by means of different solvents to afford the following three mixtures:

(a) containing mainly amino acids and other nitrogen compounds;

(b) consisting, primarily, of aromatic carboxylic acids;

(c) a mixture of flavanoids and carboxylic esters.

For the fractionation experiments additional liquid-liquid extraction techniques /9/ were also applied.

The extracts were investigated by different analytical methods and the results are shown in Table 1.

The present report deals with the details of the GC/MS studies performed with the fractions obtained from propolis and Populi gemma (Table 2).

As a convenient procedure /10/ for the examination of the carboxylic acid and flavonoid constituents, the derivatization method /11,12/ was applied. Thus carboxylic acids were converted into methyl esters and flavonoids were investigated in the form of trimethylsilyl ester derivatives.

Trimethylsilylation was found to be the most simple and efficient method of derivatization allowing a reliable identification of small quantities of the materials. The correctness of the determinations was checked by recording the spectra of the silylated standard materials.

The following four chromatograms demonstrate the separation of the aromatic carboxylic acids (in the form of methyl esters) found in various fractions of propolis and Populi gemma (chromatograms 1-4).

The GC/MS technique allowed the identification of 87 and 71 chemical components of propolis and Populi gemma, respectively, and 35 of these compounds were found to be present in the fractions of both substances.

The components of the extracts, obtained by means of distribution with organic solvents of different polarity, are shown in the following total-ion chromatograms (for explanation see Table 3.

Table 1

Analytical methods

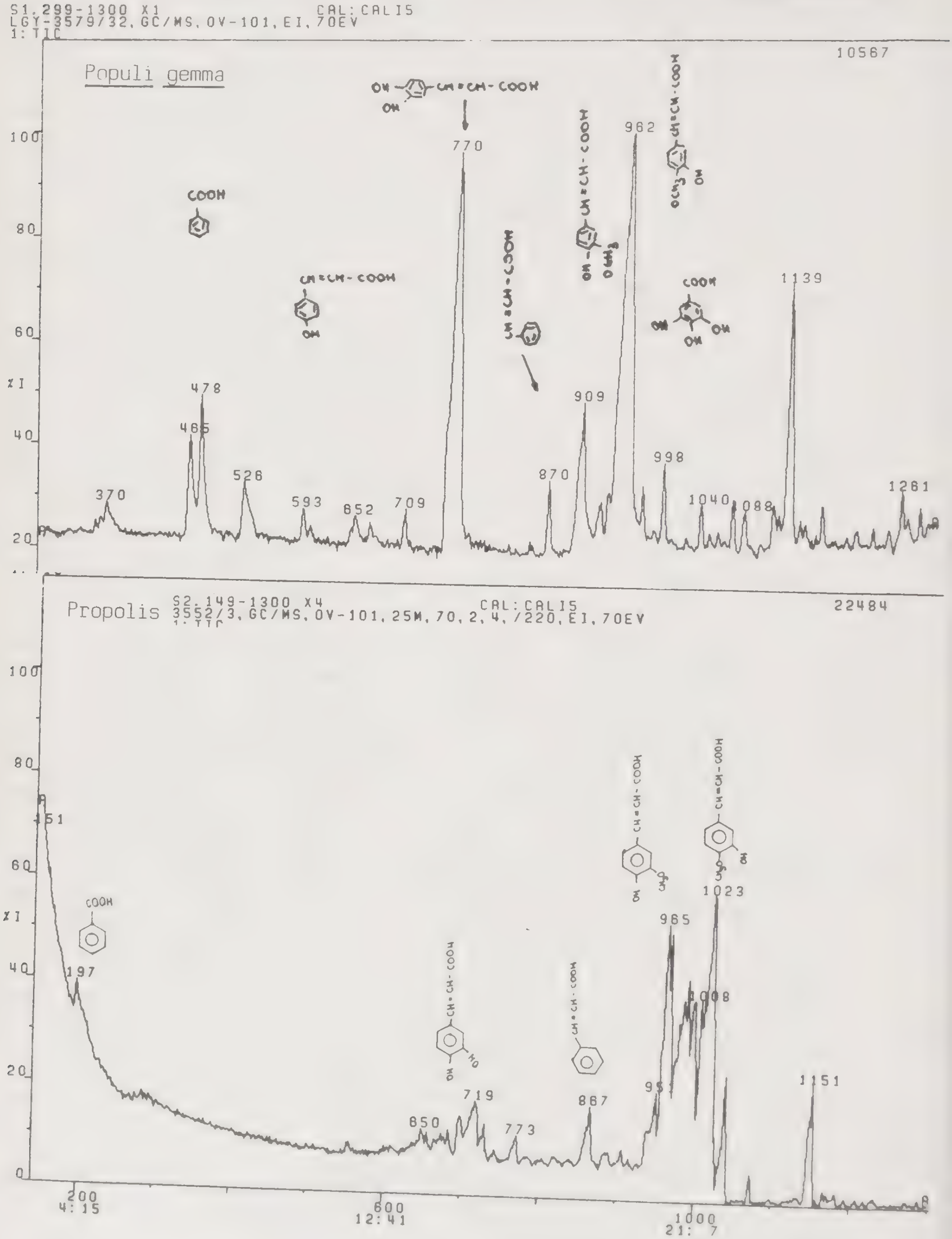
① Thin-layer chromatography TLC	② Column chromatography	③ High Performance Liquid Chromatography	④ Gas chromatography Mass spectrometry GC/MS
Preliminary identification of - flavonoids - aromatic carboxylic acids - benzoic acid derivatives - carbohydrates - amino acids - proteins - lipids - waxes - terpenoids	Isolation in crystalline form and analytical studies (UV;IR) of different components (flavonoids, aromatic carboxylic acids)	Separation and identification of aromatic carboxylic acids	1. Derivatization (TMS) 2. GC/MS EI studies of different extracts

Table 2

Gas Chromatography - Mass Spectrometry	Experimental parameters
GC: Hewlett-Packard HP-5710 A flow rate: 30 ml/min He Column: fused silica (25 m; OV-101) Injector temperature: 200 ^{o}C Temperature program: 220 ^{o}C 30' 6 ^{o}/s 100 ^{o}C 2'	MS: Mass spectrometer: VG-7035 Ionization mode: EI (+) Ion source temperature: 200 ^{o}C Accelaration voltage: 4 kV Electron energy : 70 eV

Chromatograms 1-2

Methyl ester derivatives of carboxylic acids obtained by extractions of different propolis fractions and Populi gemma



Chromatograms 3-4

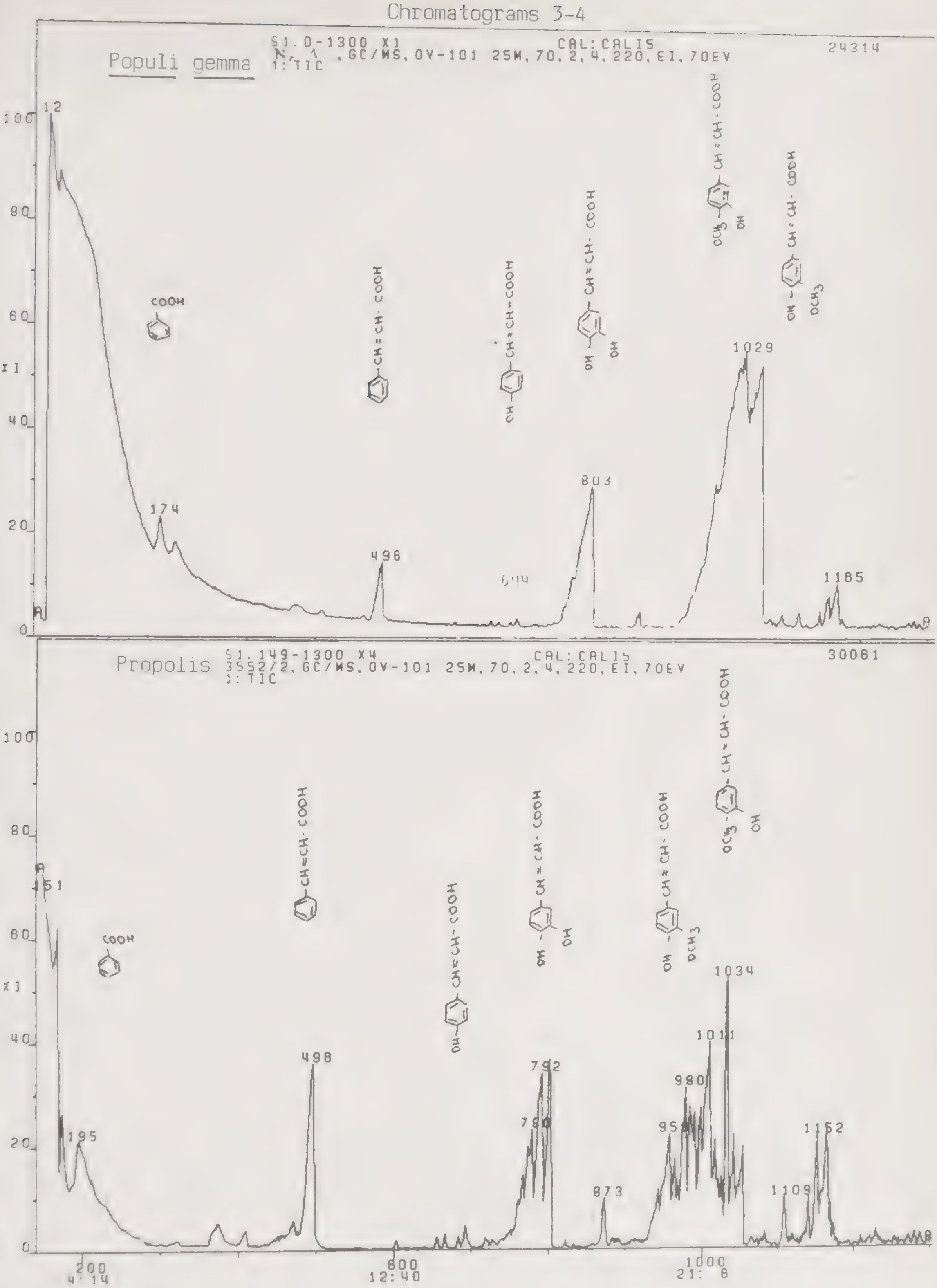


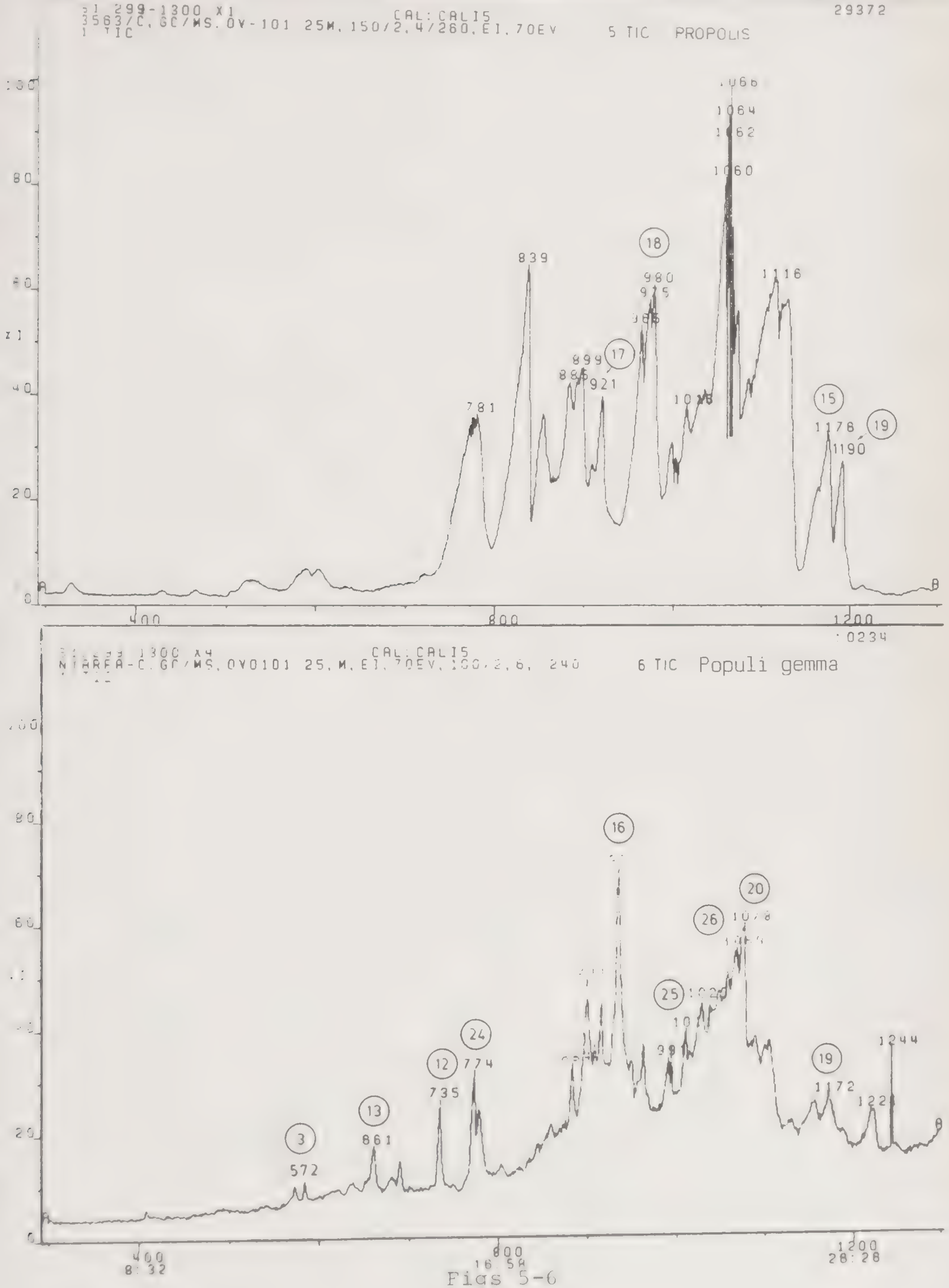
Table 3

List of compounds occurring in the total-ion chromatograms 1-12 (Figs 5-10)

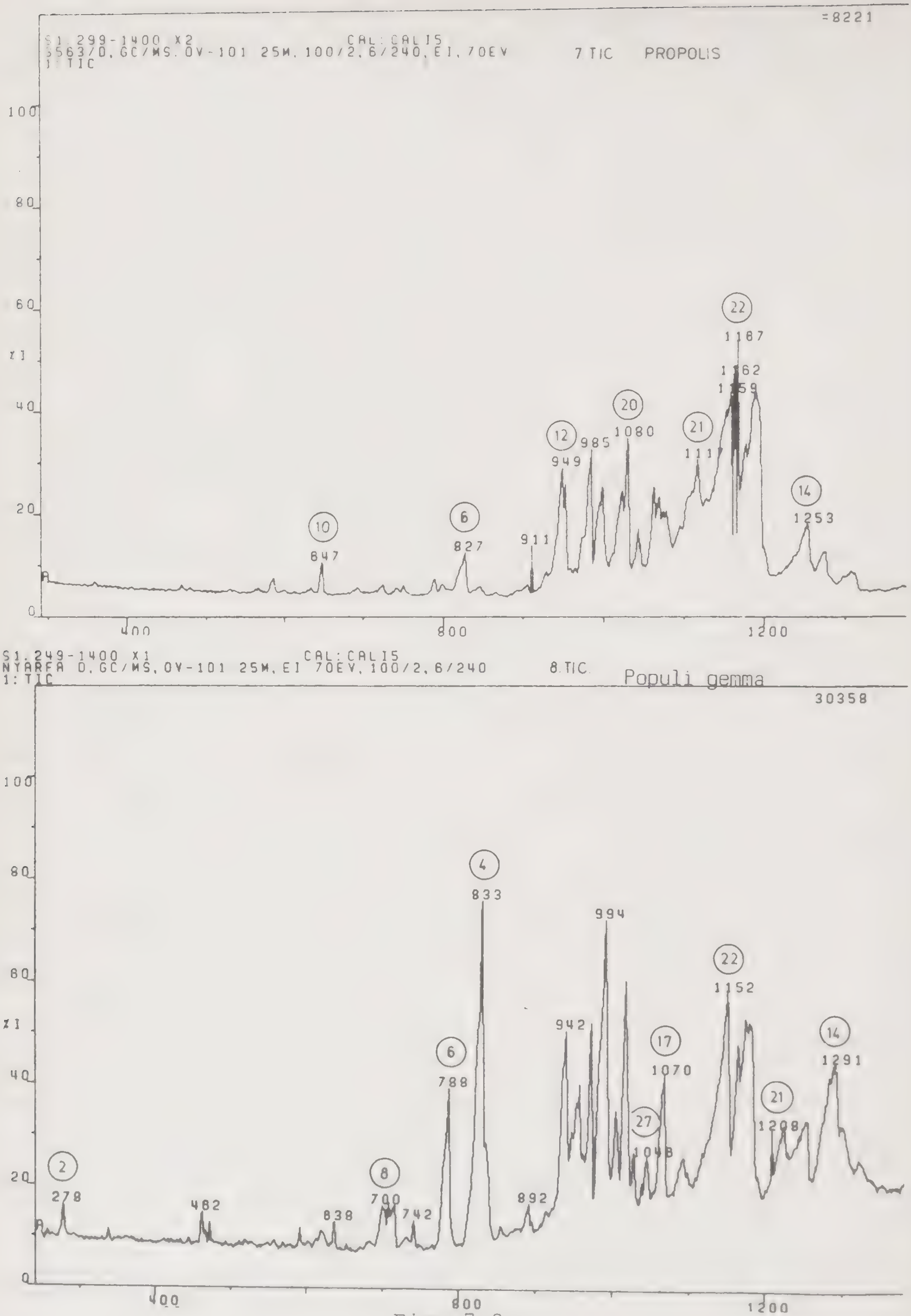
Symbol	Name	Symbol	Name
1	Benzoic acid	15	Acacetin
2	Cinnamic acid	16	Naringenin
3	MeO-cinnamic acid	17	Pectolinarigenin
4	Ferulic acid	18	Apigenin
5	Izoferulic acid	19	Hesperetin
6	Caffeic acid	20	Galangin
7	Caffeic acid phenylethyl ester	21	Rhamnocitrin
8	Vanillin	22	Pinocembrin
9	Pterostilbene	23	Kaempferol
10	Coumarin	24	Sakuranetin
11	Flavone	25	Rhamnetin
12	Chrysin	26	Izorhamnetin
13	Tectochrysin	27	3-O-acetyl-pinobanksin
14	Isalpinin		

These results clearly show that the GC/MS technique is extremely useful for the determination and identification of the components of the two native substances. It is to be noted, however, that some of the constituents identified with the GC/MS method could not be isolated by conventional separation procedures.

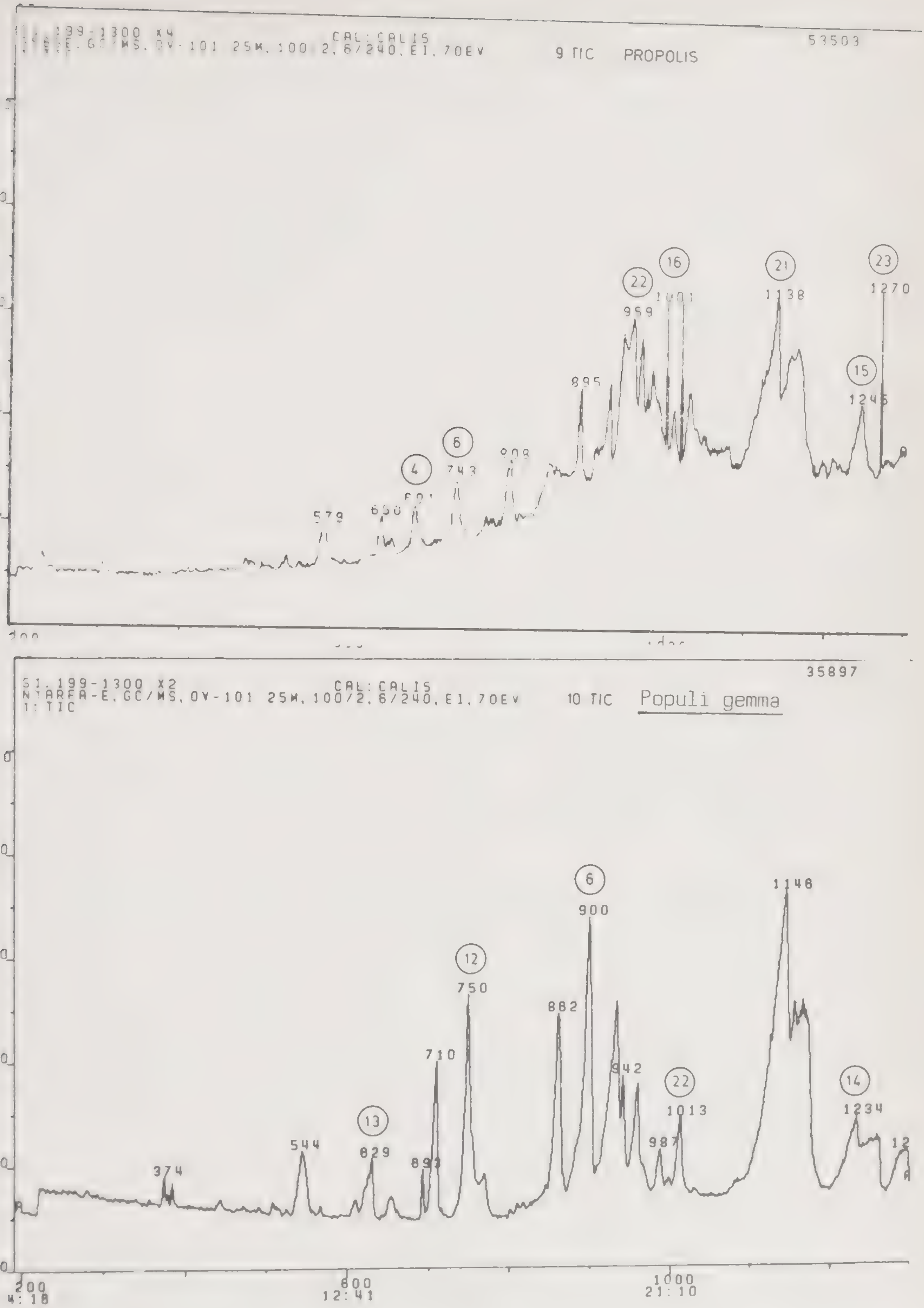
Total-ion chromatograms of sample mixtures obtained in different solvents



Figs 5-6


S1.299-1400 X2
CAL:CALIS
5563/D,GC/MS.OV-101 25M,100/2,6/240,EI,70EV
1:TIC
7 TIC
PROPOLIS
=8221
100
80
60
%I
40
20
0
400
800
1200
⑩ 647
⑥ 827
911
⑫ 949
985
⑳ 1080
㉑ 1117
㉒ 1187
1162
1159
⑭ 1253
S1.249-1400 X1
CAL:CALIS
NYARFA D,GC/MS,OV-101 25M,EI 70EV,100/2,6/240
1:TIC
8.TIC
Populi gemma
30358
100
80
60
%I
40
20
0
400
800
1200
② 278
482
638
⑧ 700
742
⑥ 788
④ 833
892
942
994
㉗ 1048
⑰ 1070
㉒ 1152
㉑ 1208
⑭ 1291

Figs 7-8


199-1300 X4
GC/MS, OV-101 25M, 100/2, 6/240, EI, 70EV
CAL: CALIS
9 TIC PROPOLIS
53503
80
20
579
650
4
6
743
808
895
22
959
16
1001
21
1138
15
1245
23
1270
S1, 199-1300 X2
NTARFA-E, GC/MS, OV-101 25M, 100/2, 6/240, EI, 70EV
1: TIC
CAL: CALIS
10 TIC Populi gemma
35897
100
80
60
%I
40
20
0
374
544
13
829
893
710
12
750
882
6
900
942
987
22
1013
1146
14
1234
12
200
4:18
600
12:41
1000
21:10

Figs 9-10

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A report on the isolation of the above-mentioned compounds is given in the following paper by V. Pápay, L. Tóth, É. Nagy, M. Soltész and Gy. Litkei.

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ISOLATED COMPOUNDS FROM HUNGARIAN PROPOLIS AND *POPULI GEMMA*

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INTRODUCTION

Natural products and preparations are today in the centre of interest. Among them are the substances produced by honey bees, which have been applied for centuries all over the world in therapy as medicinal agents.

Propolis has special significant physiological activities and it is used in dermatology for the treatment of mycotic infection, etc.

Propolis is a resinous, sticky gum. It has a complex chemical composition. Propolis contains fatty acids, waxes, volatile oils, aromatic carboxylic acids and their esters, sesquiterpenes, stilbene compounds and a great number of flavonoids in a large quantity /1-17/.

It is known that bees collect propolis from the leaves and buds of balsam trees in different geographical regions, thus the chemical composition of propolis can vary /9, 15, 17-19/.

In this paper we describe flavonoids, aromatic carboxylic acids and esters isolated from Hungarian propolis and

Populi gemma, and the pharmacological activities of these compounds.

RESULTS AND DISCUSSION

We have been dealing with the chemical and pharmacological examination of propolis and Populi gemma since 1979 in our Department in Szeged and since 1983 in Debrecen. We held lectures about the isolation of flavonoids of propolis in 1982, 1983, 1985 in Szeged, Bratislava and Debrecen /21/.

We had several targets in the examination. These were the elucidation of the chemical compositon of propolis; establishing which are the biologically active substances found in Hungarian propolis; identification of the similar substance in propolis and in balsam trees, and finding out which are substances commonly occurring in all parts of the world where bees collect propolis.

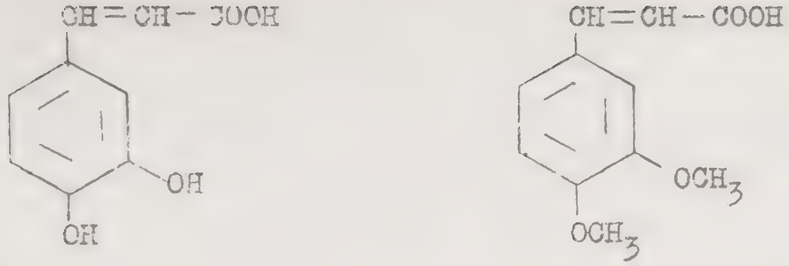
The plant origin of Hungarian propolis was examined and it was found that Hungarian propolis is collected by bees from different trees, but mainly from Populus species, especially Populus nigra.

Propolis and buds of Populus nigra were examined parallel, using the same methods.

Extraction and isolation data are shown in Table 1.

The lipoid-soluble fraction showed high antibacterial, antifungal, anti-inflammatory actions and capillary activity, hence this fractions was closely examined.

The isolated compounds are the following: the flavones shown in Table 2, flavonols in Table 3, flavonones in Table 4,



CAFFEIC ACID

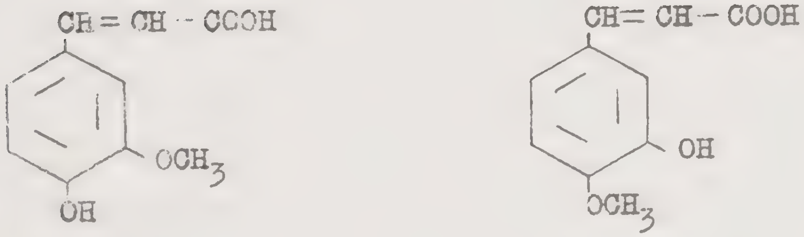
M.p. 195° C

M^+ 180

3,4-DIMETHOXYCINNAMIC ACID

M.p. 180° C

M^+ 208



FERULIC ACID

M.p. 168° C

M^+ 194

ISOFERULIC ACID

M.p. 228° C

M^+ 194

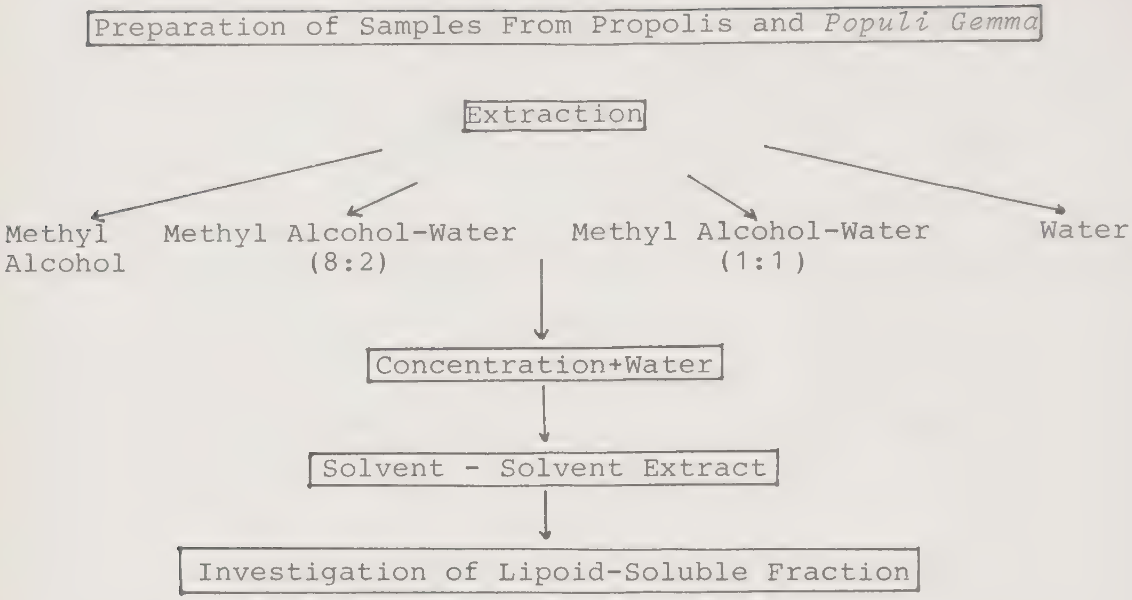
Fig. 1.

CINNAMIC ACID DERIVATIVES FROM PROPOLIS AND *POPULI GEMMA*

cinnamic acid derivatives in Fig. 1.

A large quantity of a mixture of caffeic acid derivatives was also isolated from propolis and *Populi gemma* /20/. According to the data of the mass spectrometry /M^+; 296, 284, 270/ there were three compounds present. Perhaps these substances are like those isolated by Schneidewind, identified

Table 1.


Preparation of Samples From Propolis and *Populi Gemma*
Extraction
Methyl Alcohol
Methyl Alcohol-Water (8:2)
Methyl Alcohol-Water (1:1)
Water
Concentration+Water
Solvent - Solvent Extract
Investigation of Lipoid-Soluble Fraction

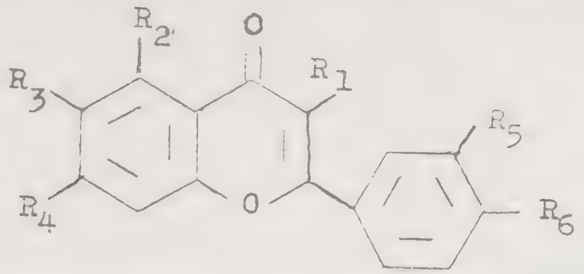
1. Silica Column Chromatography Isolation in Crystalline Form and
2. Sephadex LH-20 Column -"- Analytical Studies
3. Silica Preparative Layer -"- MP, UV, IR, Mass Spectrometry
4. Thin-Layer Chromatography of Different Flavonoids and Aromatic Carboxylic Acids

as caffeic acid cinnamyl ester, caffeic acid phenyl ethyl ester and caffeic acid benzyl ester /11/.

The m.p. of the mixture was 118-120°C. Separation on preparative silica gel layer and crystallization gave one of the components as a pure compound which was identified by mass spectrometry. It is caffeic acid phenyl ethyl ester /Fig. 2/.

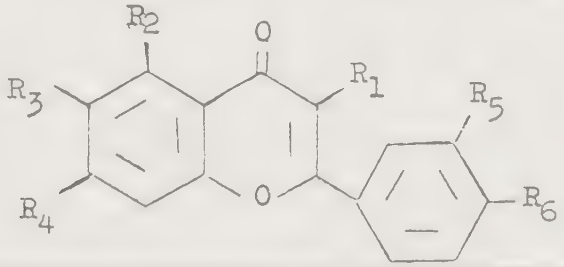
The isolated substances of propolis and *Populi gemma* were the same; 6 out of 9 flavonols, 4 out of 5 flavones, 5 out of 6 flavanones and 4 out of 4 aromatic carboxylic acids and their esters were identical.

Table 2. Flavones from Propolis and *Populi Gemma*



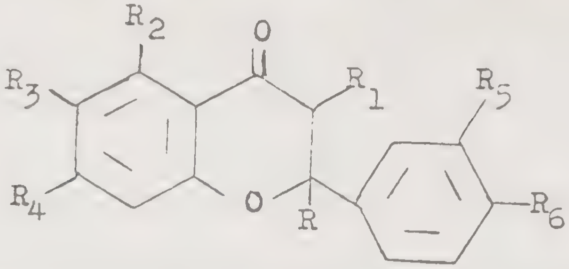
	M.P.	M^+	R_1	R_2	R_3	R_4	R_5	R_6	Propolis	*Populi Gemma*
Chrysin	288	254	H	OH	H	OH	H	H	+	+
Tectochrysin	165-66	268	H	OH	H	OCH_3	H	H	+	+
Apigenin	347-49	270	H	OH	H	OH	H	OH	+	+
Acacetin	260-62	284	H	OH	H	OH	H	OCH_3		+
5-OCH_3-3,7-OH-Flavone		284	OH	OCH_3	H	OH	H	H	+	+

Table 3. Flavonols from Propolis and *Populi Gemma*



	M.p.	M^+	R_1	R_2	R_3	R_4	R_5	R_6	Propolis	*Populi Gemma*
Galangin	216-18	270	OH	OH	H	OH	H	H	+	+
Galangin-3-OCH_3	275	284	OCH_3	OH	H	OH	H	H	+	+
Isalpinin	197	284	OH	OH	H	OCH_3	H	H	+	+
Kaempferol	278	286	OH	OH	H	OH	H	OH	+	+
Kaempferid	225-27	300	OH	OH	H	OH	H	OCH_3	+	
Rhamnocitrin	223-25	300	OH	OH	H	OCH_3	H	OH	+	
Rhamnetin	296-97	316	OH	OH	H	OH	OH	OCH_3	+	+
Isorhamnetin	305-7	316	OH	OH	H	OH	OCH_3	OH	+	+
Quercetin	310-12	302	OH	OH	H	OH	OH	OH	+	
Quercetin-3,3'-dimethyl ether	256	330	OCH_3	OH	H	OH	OCH_3	OH		

Table 4. Flavanones from Propolis and *Populi Gemma*



	M.p.	M^+	R	R_1	R_2	R_3	R_4	Propolis	*Populi Gemma*
Pinocembrin	195-96	256	H	H	OH	OH	H	+	+
Pinostrobin	111-113	270	H	H	OH	OCH_3	H	+	+
Pinobanksin	174-76	284	H	OH	OH	OH	H	+	+
3-Acetyl pinobanksin	178-80	314	H	$OCOCH_3$	OH	OH	H	+	+
5-OCH_3-3,7-OH-flavanone	218	286	H	H	OCH_3	OH	H	+	
2,5-OH-7-OCH_3-flavanone	170	286	OH	H	OH	OCH_3	H	+	+

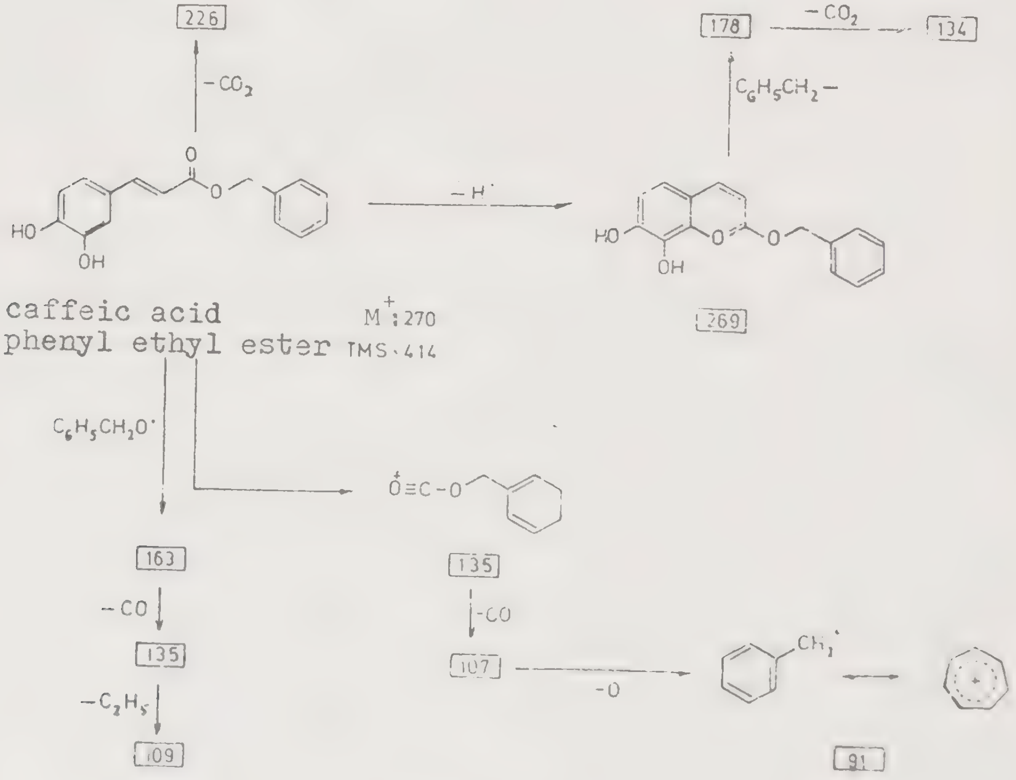


Fig. 2.

SUMMARY

4 favones, 9 flavonols, 5 flavanones, 4 aromatic carboxylic acids and 1 carboxylic acid ester were isolated from Hungarian propolis. 5 flavones, 6 flavonols, 4 flavanones, 4 aromatic carboxylic acids and 1 carboxylic acid ester were isolated and identified from Populi gemma. 6 out of 9 flavonols, 4 out of 5 flavones, 5 out of 6 flavanones, 4 carboxylic acids and caffeic acid phenyl ethyl ester are the same in propolis and Populi gemma.

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(Eds. L. Farkas, M. Gábor, F. Kállay)

POLYPHENOLS OF *COTINUS COGGYGRIA* AND THEIR THERAPEUTICAL VALUE

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INTRODUCTION

Cotinus coggygria is a tannin plant and contains these compounds in 5-15% in different organs, the quantity of which depends on the environmental factors too /1/. A review was given by us about our results in this field at the Medicinal Plant Conference in the German People's Republic /2/.

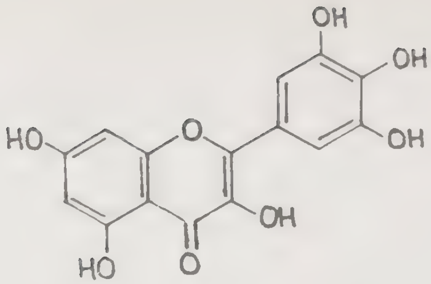
The present aim of our work was to discover relations between the chemical composition and therapeutical value. For this purpose we prepared four extracts.

METHODS

The methodology of the chemical processes is not disclosed for patent reasons. In general UV-VIS, IR and NMR spectroscopy, as well as TLC CL methods were used /3/. The microbiological technique was a filter paper disk method, later on a sheet-method with solubilisation. The virological investigations were made with Influenza A (PR 8/34) and B (Lee/40) types; 10^{-9} conc. dilution was prepared and to this solution 10^{-1}, 10^{-2} and 10^{-3} diluted Cotinus extracts (Fraction 4) were given, working with eggs at 37°C temperature and using 48 hours incubation time.

RESULTS

The 1st extract was an aquous methanolic one. On the basis of spectroscopical and thin-layer chromatographic investigations, in this fraction gallic acid, m-digallic acid and glycogallic compounds were detected. Myricetin glycoside is also present (Fig. 1).



myricetin

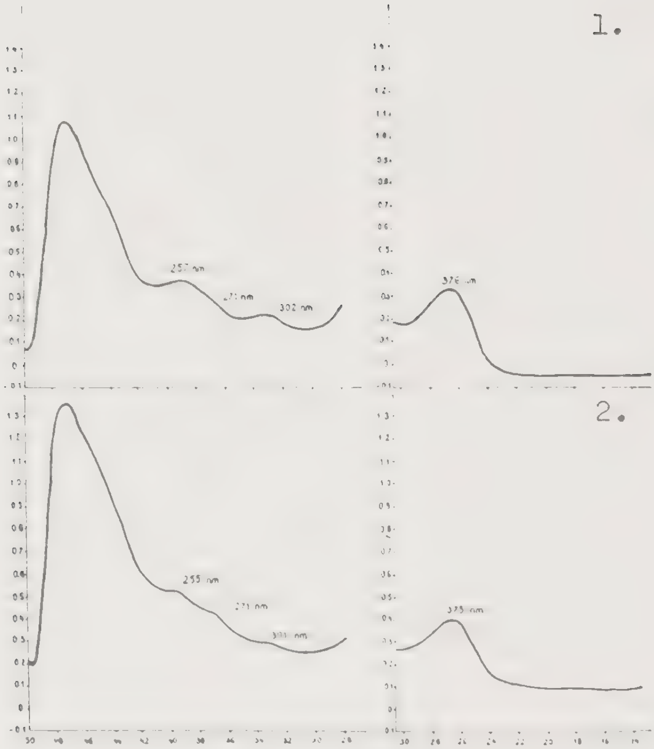


Fig. 1. Comparison of myricetin and test material using UV spectroscopy

1. Myricetin isolated from the extract
2. Myricetin compound

The tannin compounds were separated from the waxy and tarry components by extraction with organic solvents and using precipitation in the next step. In this way a crude extract (Fraction 2) was obtained; purification gave a precipitate containing tannins and flavonoids in the proportion 5:4 (Fraction 3). In this product the flavonoids were present in glycosidic form. The flavonoid glycoside content is increased in this preparation from 0.2% contained in the plant (Fig. 2).

Investigation of the 4th fraction, which seems to be the residue of the total extract, revealed that this fraction contained a main component, which was a procyanidin.

Further chemical evaluation of the 4th fraction showed that the main component could readily be transformed to anthocyan by treatment with 2N hydrochloric acid (Fig. 3). In this fraction there are some gallotannins present, too, which interfere with the detection. This fraction was purified by extraction with ethyl acetate, drying of the solution over Na_2SO_4, and adding benzene to it, whereupon the gallotannins were precipitated and the procyanidin remained alone in solution. After this procedure it was seen that the material also contained catechin (Fig. 4). So the effective substance seems to be a dimeric procyanidin molecule. More thorough structure elucidation is in progress.

To establish the terapeutical value, our fractions were subjected to pharmacological and chemical investigation.

1. The first fraction showed a healing effect against different sorts of gingivitis. This fraction contains gallic acid derivatives as the main components.

2. The second and the purified third fraction, which contain tannins and flavonoids in 5:4 proportion, has a significant and lasting effect increasing the capillary resistance (Fig. 5). This therapeutical effect is more marked than that of Venoruton and lasts for 12 hours; the effect is also higher than that of the first extract (Tables 1, 2).

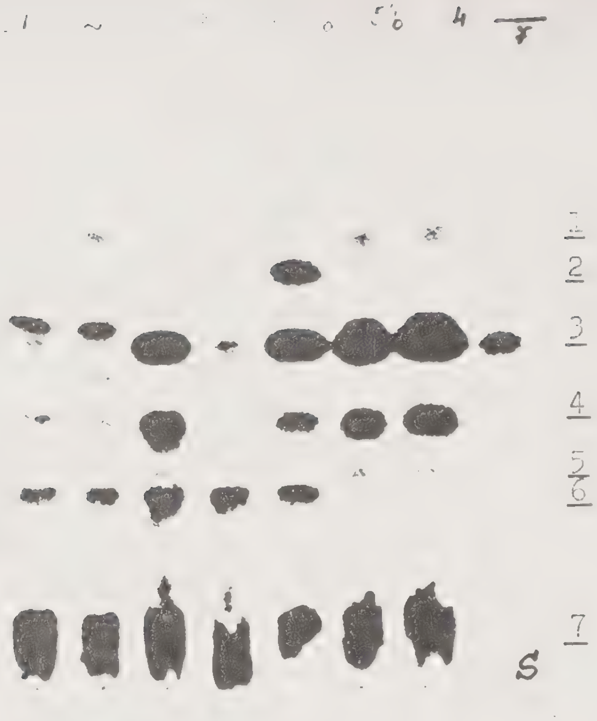


Fig. 2. Thin-layer chromatography of tannins and flavonoids. Layer: Kieselgel Merck, 0.25 mesh; solvent system: ethyl acetate, chloroform, formic acid, water 82:8:5:5; reagent: 2% $FeCl_3$ in methanol. R_f values: gallic acid 0.70; m-digallic acid 0.60; myricetin 0.40; gallotannin 0.2.
Thin-layer chromatogram of tannins (1-4,7) and flavonoids (5,6)

3. The most important effect was found in the fourth fraction containing the procyanidin. We established that this fraction has both antiviral and antibacterial effects (Tables 3 and 4). The extract was applied for the treatment of different throat complaints (Table 5) and showed a better healing effect than medicaments in general use (Table 6).

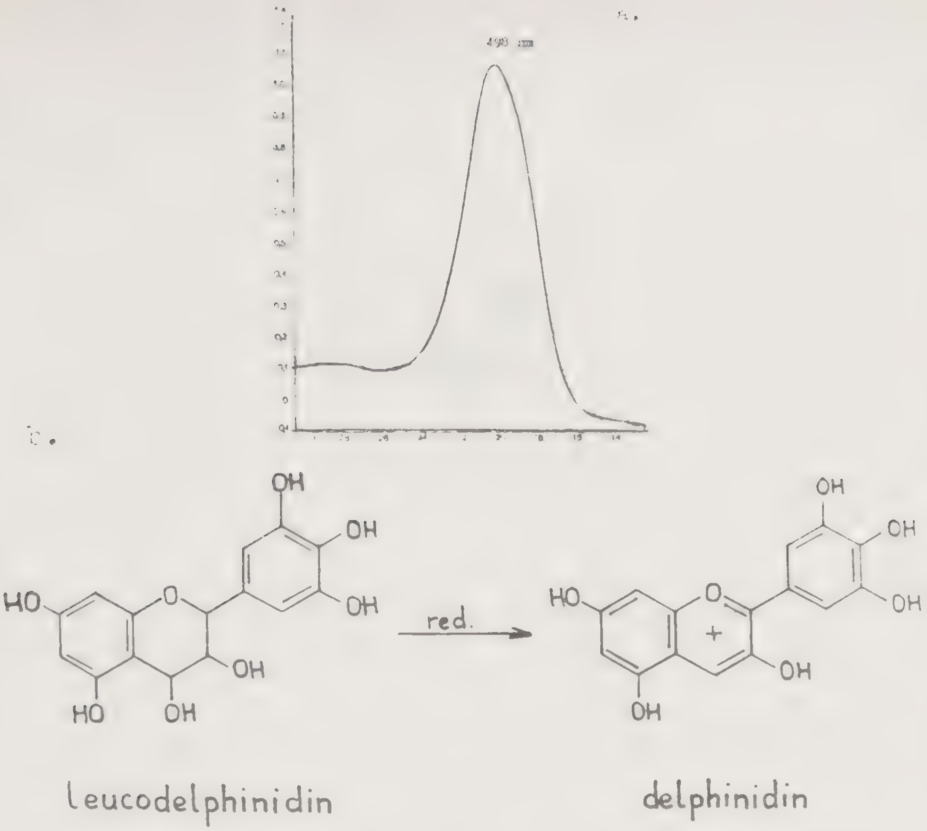


Fig. 3. VIS-spectrum of anthocyan formed from procyanidin (a) and its structure (b)

Table 1. Effect of Venoruton on capillary resistance
Dose: 50 mg/kg i.p.

Serial number of animals	Weight of rats: 300-350 g	CR/Hg cm after the treatment, in hours				
		2	4	6	8	24
1	10	25	15	10	10	10
2	10	15	10	10	10	10
3	10	10	10	15	15	15
4	10	10	10	15	10	10
5	10	20	10	10	15	10
6	10	15	15	10	10	10
7	10	10	15	10	10	10
8	10	20	15	10	10	10
$\bar{x}$	10.00	15.62	12.50	11.25	11.25	10.62
S_x	0	5.62	2.67	2.31	2.31	1.76
t		2.82	2.64	1.52	1.52	1.00
p		0.05	0.05	0.05	0.05	0.05

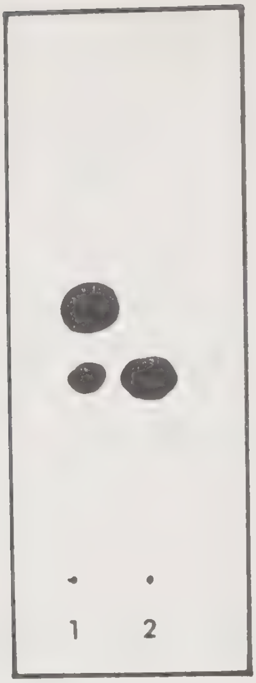


Fig. 4. Thin-layer chromatogram with catechin and flavan spot (1); procyanidin (2)

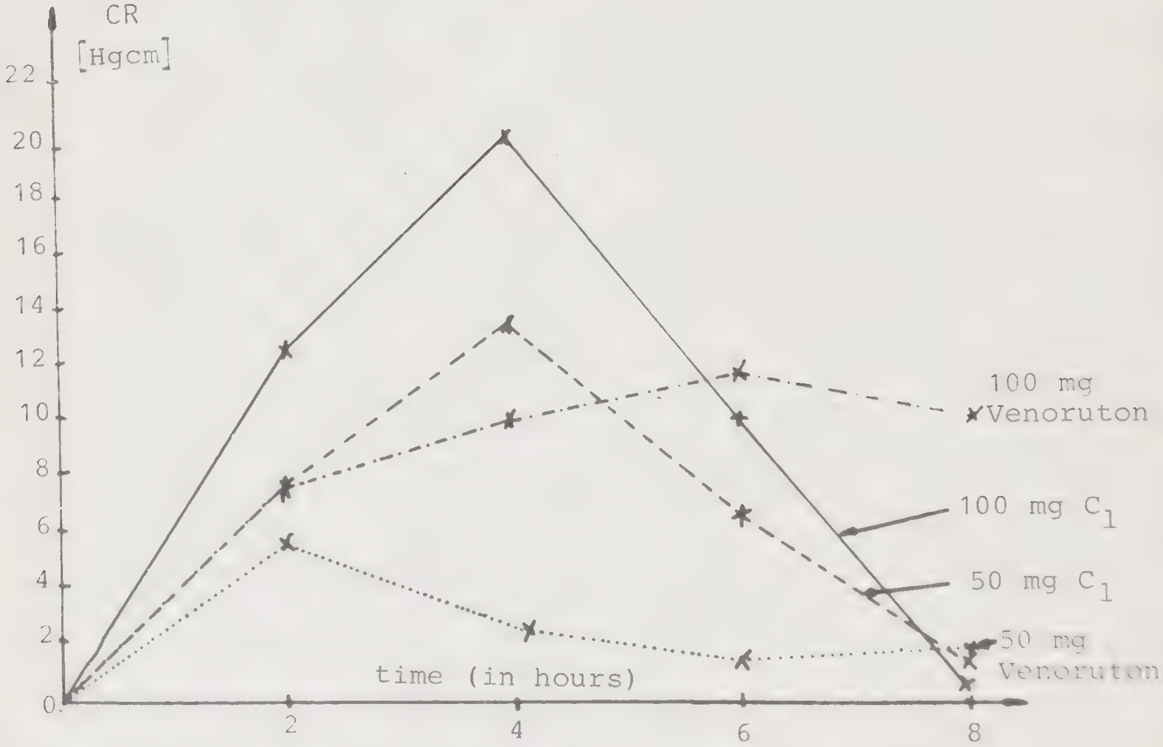


Fig. 5. Comparison of Cotinus extract (Fraction 1) with Venoruton: capillary resistance

Table 2. Effect of *Cotinus* extract (Fraction 2) on capillary resistance
Dose: 50 mg/kg i.p.

Serial number of animals	CR/Hg cm after the treatment in hours							
	0	2	3	4	6	8	10	12
1	10	20	30	25	25	25	20	20
2	10	20	30	35	30	25	25	20
3	15	20	30	25	25	25	20	20
4	10	20	30	30	30	30	30	25
5	10	20	30	25	25	25	20	20
6	10	20	25	25	20	20	20	15
$\bar{x}$	10.83	20.0	29.17	27.50	25.83	25.00	22.5	20.0
S_x	2.04	0	2.04	4.18	3.76	3.16	4.18	3.16
t	–	11.0	17.4	7.9	8.2	9.2	5.5	5.96
p	–	0.001	0.001	0.001	0.001	0.001	0.01	0.01

Table 3. Microbiological effect of *Cotinus* extract (Fraction 4)

Bacteria	Number of sorts	Number of inhibited sorts
Staphylococcus aureus	18	11
Streptococcus pneumoniae	8	8
Streptococcus haemophyl.	5	4
Streptococcus haemophyl.	5	2
Haemophyl. influenzae	5	1
Pseudomon. aeruginosa	18	10
Proteus mirabilis	8	7
Escherichia coli	25	15
Klebsiella group	10	3

Table 4. Antiviral effect of Cotinus extract (Fraction 4)

Material	EID_{50}
Virus control	10^{-9}
Influenza A	10^{-9}
Influenza B	10^{-9}
Solvent control	10^{-9}
Cotinus + Influenza A	
10^{-1} conc.	$10^{-4.5}$
10^{-2} conc.	10^{-5}
10^{-3} conc.	$10^{-5.5}$
Cotinus + Influenza B	
10^{-1} conc.	$10^{-4.5}$
10^{-2} conc.	$10^{-4.7}$
10^{-3} conc.	$10^{-5.5}$
Tannin and flavonoid mixture + + Influenza A and B	10^{-3}

Table 5. Effect of *Cotinus* extract (Fraction 4) in clinical cases

Illness	Cured	Unchanged	Total in		Swallowing pain		Fever	
			Number	%	+	-	+	-
Pharingitis with normal throat flora	28	9	37	76	37	-	22	15
Pharyngitis with pathogen bacteria	10	4	14	71	10	4	8	6
Stomatitis	7	4	11	64	8	3	4	7
Tonsillitis acuta	20	13	53	61	33	-	30	3
Laryngitis	2	5	7	28	5	2	3	4
Total	67	35	102		93	9	67	35

+ cured; - unchanged; ++ from 14 cases there were 8 *Streptococcus* (α:5 β:3), 3 *Staphylococcus* and 3 *Pneumococcus* infections

Table 6. Curative effect of *Cotinus* extract (Fraction 4)

Illness	Glycosept	Phlogosol	Faringosept	*Cotinus coggygria*
Pharyngitis with normal throat flora	33	21	27	76
Pharyngitis with pathogen bacteria	33	21	27	71
Stomatitis	14	19	22	64
Tonsillitis acuta	13	25	20	61
Laryngitis	20	0	0	18

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Flavonoids and Bioflavonoids, 1985
(Eds. L. Farkas, M. Gábor, F. Kállay)

NEW FLAVONOL GLYCOSIDES FROM *MESPILUS GERMANICA* L.

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INTRODUCTION

Medlars (*Mespilus germanica* L., Rosaceae) and foodstuffs prepared from them (juices, jams and refreshment drinks) show a beneficial influence on the digestion, as well as on the biliary, kidney and liver functions /1/. Organic acids, monosaccharides and sorbitol /2/, as well as a glucoside of mevalolactone /3, 4/ have been isolated from this plant. In Bulgarian folk medicine medlar leaves are used in the treatment of uremia, but this application is not confirmed by literature data. This effect, however, may be connected with the presence of some polyphenolic substances in the plant, which have a hypoazotemical action. Recently, Bulgarian authors /5/ reported about some flavonol glycosides, characterized by means of acid hydrolysis and UV spectra, though the structures have not been elucidated. In the present investigation, using polyamide column chromatography and preparative paper chromatography, we have isolated six flavonoid compounds from the leaves of *Mespilus germanica*, which were identified mainly by spectral methods.

METHOD AND RESULTS

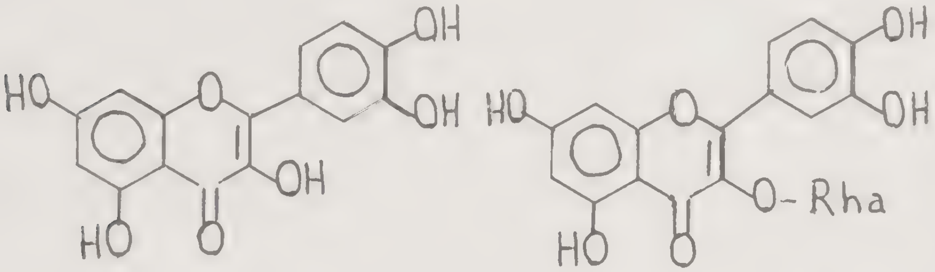
Dried ground leaves of *Mespilus germanica* were fully extracted in a Soxhlet apparatus with methanol. The extract was concentrated in vacuum, and the syrup obtained was rubbed with water. Chlorophyll and some other components which thus precipitated were separated by filtration. The clear filtrate was first extracted with chloroform and then with n-butanol. The residue after concentration of the butanol extract was dissolved in a minimum of aqueous methanol and the solution was carefully applied to a polyamide column. Elution was started with water, followed by 30 and 50% aqueous ethanol and, finally, methanol. From the fractions obtained with 30% ethanol compound (3) crystallized on evaporation to dryness and dissolution in hot ethanol. Compound (1) was obtained from the 50% ethanol fractions. The mixed fractions (water, 30% ethanol) were further separated by preparative paper chromatography in BAW (4:1:2) and 15% HOAc solvent systems. The separation in the two systems was repeated until chromatographically pure zones had been obtained. Evaporation of the pure fractions and further purification by recrystallization from the solvents shown in Table 1 gave the crystalline compounds (2),(4) and (5), and the amorphous compound (6). Data of the isolated compounds are given in Table 1.

Table 1. Data of flavonoids isolated from *Mespilus germanica*

Compound	Formula	M.p., °C	Yield, %	R_f-values in solvent systems "a"	"b"
(1)	$C_{15}H_{10}O_7$	310-312 (50% EtOH)	0.0025	0.78	0.03
(2)	$C_{21}H_{20}O_{11}$	181-182 (Acetone/H_2O)	0.007	0.72	0.57
(3)	$C_{21}H_{20}O_{12}$	216-218 (EtOH)	0.11	0.55	0.33
(4)	$C_{27}H_{30}O_{16}$	189-192 (MeOH/H_2O)	0.089	0.35	0.48
(5)	$C_{27}H_{30}O_{15}$	190-192 (Acetone/H_2O)	0.127	0.50	0.58
(6)	$C_{29}H_{32}O_{16}$	160-162 (EtOH, amorph.)	0.065	0.65	0.68

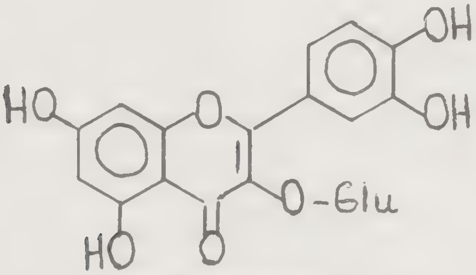
"a": n-Butanol-Acetic acid-Water (4:1:2);
"b": 15% Acetic acid.

Compounds (1), (2) and (3) could be identified as quercetin, quercetin-3-α-L-rhamnopyranoside (quercitrin) and quercetin-3-β-D-glucopyranoside (isoquercitrin), respectively, by means of mixed melting point, co-chromatography and IR spectroscopy, using authentical samples as references (Fig. 1).



Quercetin (1)

Quercitrin (2)



Isoquercitrin (3)

Fig. 1. Formulas of compounds (1), (2) and (3)

The chromatographical and UV data before and after hydrolysis show, that compounds (4), (5) and (6) must be related to quercetin-3,7-diglycosides with free 5, 3′ and 4′-hydroxyl groups. The UV data are given in Tables 2 and 3.

Compound (4) with m.p. 189-192^{o} yielded on acid hydrolysis quercetin, glucose and rhamnose. The distribution of the carbohydrate components can directly be determined from MS of the permethylated product (Fig. 2). The sugar at C-7 could always be identified by the higher relative intensity of the A + H + monosaccharide fragment /6/. Since in MS of compound (4) the A + H + Rha-peak and the A + H + Glu-peak appear at 532 (rel.

Table 2. UV spectral data of compounds (4), (5) and (6)

Compound (4): (MeOH) λ_{max} = 256, 268 (sh), 358 nm; (+NaOAc) 264, 412; (+H_3BO_3) 261, 381; (+$AlCl_3$) 275, 303 (sh), 340, 442; ($AlCl_3$/HCl) 270, 300 (sh), 365 (sh), 406.

Compound (5): (MeOH) λ_{max} = 255, 266 (sh), 350 nm; (+NaOAc) 264, 397; (+H_3BO_3) 259, 371; (+$AlCl_3$) 274, 302 (sh), 335, 441; (+$AlCl_3$/HCl) 272, 300 (sh), 365, 403.

Compound (6): (MeOH) λ_{max} = 255, 266 (sh), 351 nm; (+NaOAc) 263, 392; (+H_3BO_3) 258, 366; (+$AlCl_3$) 232 (sh), 274, 305 (sh) 335, 441; (+$AlCl_3$/HCl) 272, 300 (sh), 363, 404.

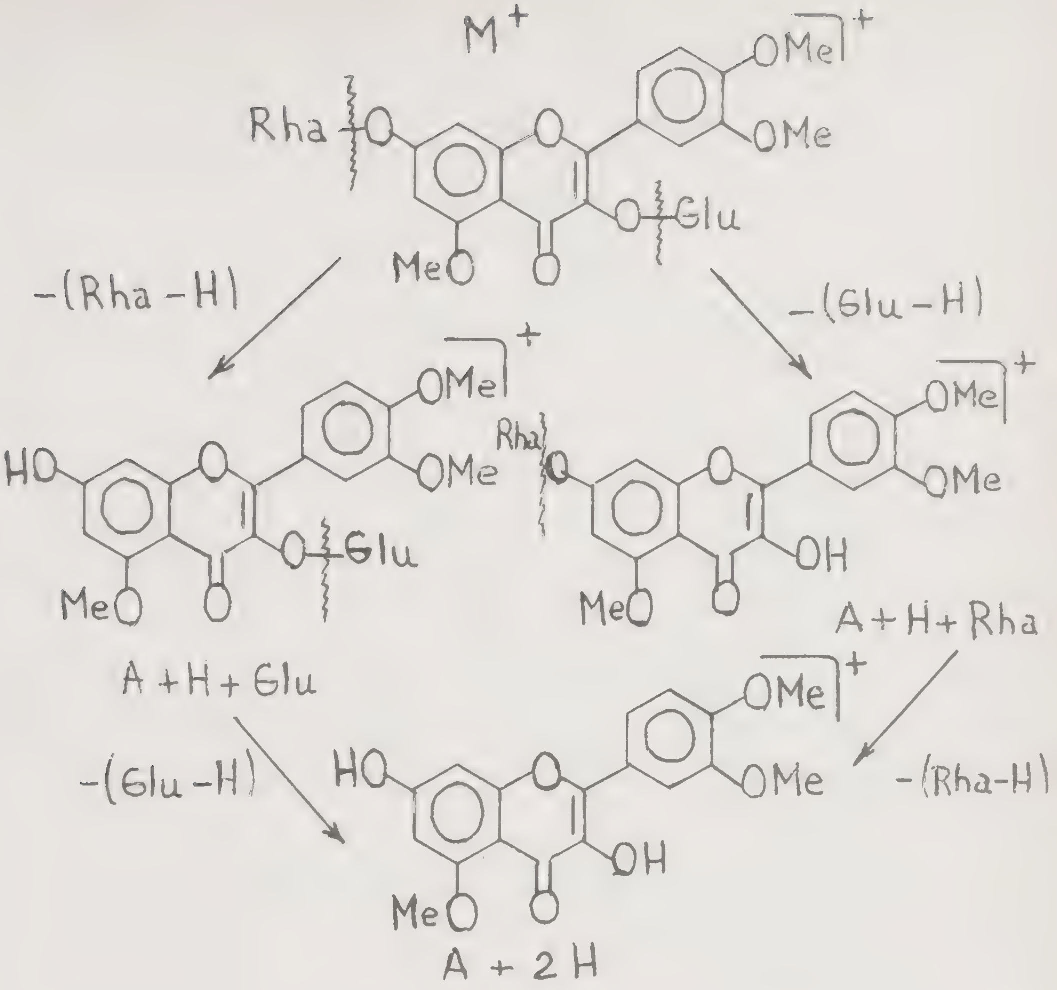
Table 3

Compound	OH-3′, 4′		OH-5
	Bathochromic shift with NaOAc/H_3BO_3 (nm) relative to MeOH spectrum (Band I)	Bathochromic shift with $AlCl_3$ (nm) relative to $AlCl_3$/HCl spectrum (Band I)	Bathochromic shift with $AlCl_3$/HCl (nm) relative to MeOH spectrum (Band I)
(4)	23	36	48
(5)	21	38	53
(6)	15	37	53

int. 23%) and 562 (rel. int. 0.4%), resp., compound (4) must be identified as quercetin-3-gluco-7-rhamnoside.

The full structure of compound (4) can be determined as quercetin-3-β-D-glucopyranosido-7-α-L-rhamnopyranoside /8,9/ from the NMR and mass spectra of its peracetate /7/(Fig. 3).

Compound (5) with m.p. 190-192° yielded on acid hydrolysis quercetin and rhamnose, the latter being found, according to the UV spectra, to be in positions 3-0 and 7-0. MS of the permethyl- and the NMR spectrum of the peracetyl derivative (Fig. 4) were interpreted in the same way to a correct identi-

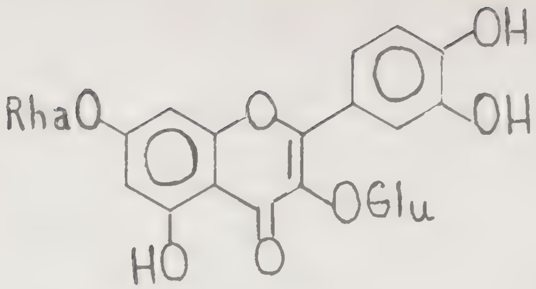


MS (PME): m/e 750 M^+ (<0.1% rel. int.), 562 A + H + Glu (0.4), 532 A + H + Rha(23), 344 A + 2H(100) 315(7) 298(15) 218(20) 189(19) 188(22) 187(8) 165(15) 157(12) 155(6) 145(10) 125(11) 116(74) 111(22) 101(70)

Fig. 2. Fragmentation and MS of the permethylated product of compound (4)

fication of compound (5) as quercetin-3,7-di-α-L-rhamnopyranoside /10/.

Compound (6) with m.p. 160-162° yielded on acid hydrolysis also quercetin and rhamnose and according to its UV spectra it turned out to be a quercetin-3,7-dirhamnoside. Since there is an ester band found at 1745 cm^{-1} in the IR spectrum (Fig. 5), we



Quercetin-3-β-D-glucopyranosido-7-α-L-rhamnopyranoside (4)

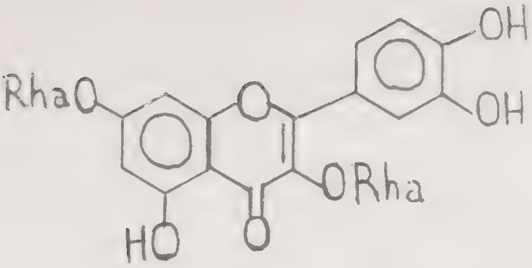
Peracetate. H-NMR: ($CDCl_3$, TMS int.) δ = 7.99 ppm (m, 2H) H-2′, 6′ 7.35 (d, J=9.5, 1H) H-5; 7.13 (d, J=2.5, 1H) H-8; 6.80 (d, J=2.5, 1H) H-6; 5.78-4.95 (m, 8H) CH-1,2,3,4 Glu + Rha; 4.10-3.50 (m, 4H) CH_2-6 Glu, CH-5 Glu + Rha; 2.47, 2.37, 2.34 (s, 9H) OAc-5, 3′, 4′; 2.21, 2.11, 2.06, 2.04, 1.92 (s, 21H) OAc Glu + Rha; 1.25 (d, J=6.5, 3H) CH_3-Rha-7.

Peracetate. MS: m/e 700 (2% rel. int.) A + H + Rha, 658(3) 616(1) 574(1) 428(5) A + 2H, 386(7) 344(14) 331(24) Glu, 302 (52) 273(53) Rha, 213(7) 171(24) 169(100) 153(72) 145(10) 139(12) 127(28) 113(23) 111(97) 109(65) 43(> 100)

Fig. 3. H-NMR-spectrum and MS of the compound (4)-peracetate

carried out a saponification with 1% NaOH. The acetic acid was detected as hydroxamate in the hydrolysate by paper chromatography. As the mass spectrum of the permethyl ether (Fig. 5) shows rhamnose fragments at m/e 189 and 217, one acetyl group should be attached to one of the rhamnoses. If the acetyl group were affixed to the 7-O-rhamnose, an intensive peak should appear at m/e 560. Only one peak is, however, found at m/e 532. This shows unambiguously an acetylated 3-O-rhamnose.

T-fragments appear in the MS of the IP-PM derivative /11/ at m/e 201 and 229 (Fig. 6) accompanied by a weak M^+ peak at m/e 772. That means both sugars are isopropylated. Analogously to the PM-derivative, the peak at m/e 544 is rather strong, while the peak at m/e 572 cannot be found at all. The acetyl group can therefore be situated only in the position 4"-O of



Quercetin-3,7-di-α-L-rhamnopyranoside (5)

MS (PME): *m/e* 720 M^+ (<0.1% rel. int.), 532 A + H + Rha (20), 500(7) 468(8) 411(11) 344 A + 2H(100) 315(10) 298(38) 189(15) 188(17) 165(27) 157(4) 125(12) 116(23) 101(30)

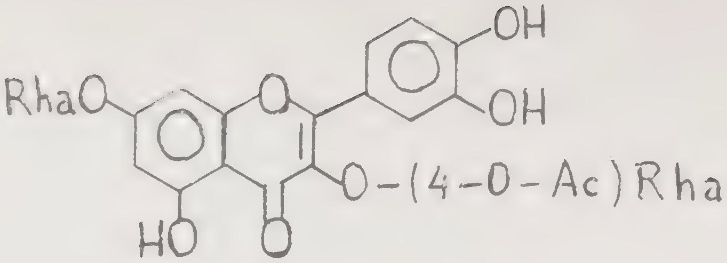
Peracetate. H-NMR: ($CDCl_3$, TMS int.) δ = 7.84 ppm (m, 2H) H-2′, 6′ 7.40 (d, *J*=9 Hz, 1H) H-5; 7.10 (d, *J*=2.5, 1H) H-8; 6.81 (d, *J*=2.5, 1H) H-6; 5.75-4.75 (m, 8H) CH-1,2,3,4 Rha; 3.96 (m, 1H) CH-5 Rha-7; 3.41 (m, 1H) CH-5 Rha-3; 2.45 (s, 3H) OAc-5; 2.32 (s, 6H) OAc-3′,4′; 2.20 2.13, 2.08, 2.05, 2.00 (s, 18H) OAc-Rha; 1.26 (d, *J*=6.5, 3H) CH_3-Rha-7;0.91(d, *J*=6.5, 3H)CH_3-Rha-3.

Peracetate. MS: *m/e* 700 (2% rel. int.) A + H + Rha-7, 658(1) 616(1) 574(1) 428(3) A + 2H, 386(5) 344(13) 302(60) 273(65) Rha, 231(8) 213(10) 171(32) 153(95) 129(42) 113(30) 111(100) 43(> 100).

Fig. 4. MS of the permethyl derivative and H-NMR spectrum of the peracetate of compound (5)

the 3-O-rhamnose. Hence compound (6) has the structure of a quercetin-3-α-L-(4-O-acetyl)-rhamnopyranosido-7-α-L-rhamnopyranoside. This latter compound has not been described in the literature till now.

All flavonoids isolated by us from *Mespilus germanica* are presented in Fig. 7.



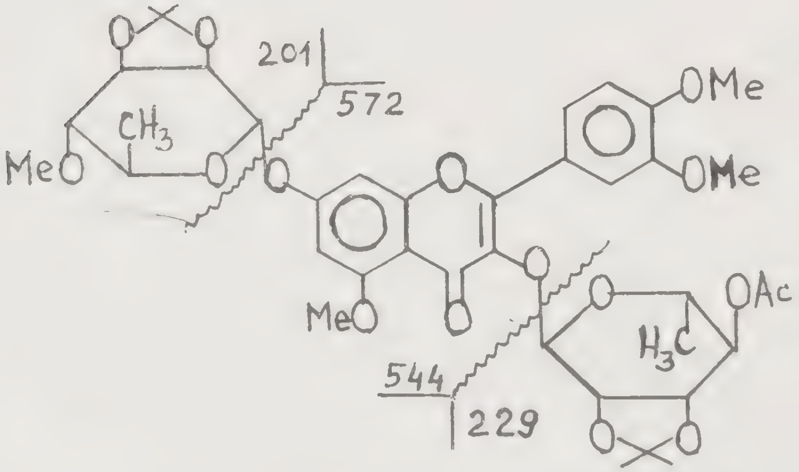
Quercetin-3-α-L-(4-O-acetyl)-rhamnopyranosido-
7-α-L-rhamnopyranoside (6)

IR (KBr): $\bar{\nu}$ = 3400 cm^{-1} (OH), 1745 (ester-C=O), 1650 (flavone-C=O).

MS(PME): m/e 532(22% rel. int.) A + H + Rha, 345(25) 344 A + 2H(100) 315(14) 314(22) 298(9) 217(6) 189(17) 188(18) 165(11) 157(20) 129(11) 125(14) 116(28) 101(44).

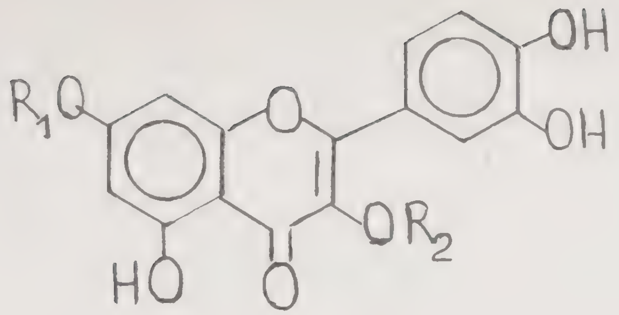
Peracetate. MS: the same as MS of compound (5)-PA

Fig. 5. IR spectrum of compound (6) and MS of its permethyl ether



Diisopropylidene permethyl ether. MS: m/e 772 (0.1% rel. int.) 544(17) 484(2) 425(4) 345(24) 344 A + 2H(100) 315(14) 298(9) 229(12) 201(11) 200(11) 165(10) 143(12) 129(18) 101(23) 99(17) 85(31) 59(29) 43(45)

Fig. 6. MS of di-IP-PM derivative of compound (6)



(1) $R_1 = R_2 = H$;
(2) $R_1 = H$, $R_2 = Rha$;
(3) $R_1 = H$, $R_2 = Glu$;
(4) $R_1 = Rha$, $R_2 = Glu$;
(5) $R_1 = R_2 = Rha$;
(6) $R_1 = Rha$, $R_2 = 4\text{-O-Ac-Rha}$.

Fig. 7. Flavonoids from *Mespilus germanica*

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IDENTIFICATION OF THE ANTHOCYANIN OF *CONSOLIDA ORIENTALIS* SCHRÖD

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INTRODUCTION

The structure of the anthocyanin which gives the blue flower-colour of two related species of Consolida orientalis (Gay.) Schröd. (Helleboraceae) - Eastern larkspur - the Consolida regalis S.F. Gray and Consolida aiacis (L.) Schur, is known to be delphinidin-3,5-diglucoside /1,2/. As the flower of Consolida orientalis (Gay.) Schröd. is available in commerce as a drog by the name Calcatrippae floss, the purpose of our study was to clarify the type of the anthocyanin present in this plant.

MATERIALS AND METHODS

Extraction

The dried and powdered flowers of the C. orientalis were extracted with a solution of 0.03% HCl in methanol at room temperature for 24 hours. The extract was evaporated in vacuum at 40°C.

Purification and isolation

The evaporation residue was dissolved again in water and filtered. Thus the extract was rid, in part, of the fatty components, being less soluble in water. After this, the solution was extracted with ethyl acetate several times in order to achieve further purification. Ethyl acetate was removed from the aqueous solution in vacuum at room temperature. The next purification step was to inject the extract onto a column of Amberlite XAD-7 (3.5 x 50 cm) and to wash the column with distilled water, until the water passing through remained colorless. The column was then eluted with 0.033% HCl in MeOH, and the eluate was evaporated in vacuum at 40°C. The residue was dissolved in MeOH containing 5% acetic acid, and passed through a column (3.5 x 80 cm) containing Sephadex LH-20 organophilic gel.

The only fraction with violet colour was evaporated again in vacuum at 40°C, and separation of the residue was repeated on Sephadex LH-20 gel twice more. On evaporation and recrystallization from MeOH, the violet fraction gave dark-violet crystals.

IDENTIFICATIONS BY TLC

Identification of the anthocyanidin

Part of the anthocyanin obtained during the preceding procedure was hydrolyzed for 1 h in 1 N HCl at 100°C and then extracted with *n*-amyl alcohol. The organic phase was applied to cellulose layer (Merck 5716) and developed in a mixture of *n*-amyl alcohol:acetic acid:water (2:1:1 v/v) and formic acid: hydrochloric acid:water (10:1:3 v/v). According to the R_f values, the product was delphinidin /3/.

Identification of the sugar components

The aqueous phase containing the sugars was concentrated in vacuum at 60°C, applied to silica gel layer (Merck 5721), and then developed against authentic sugar samples in a mixture of ethyl acetate:formic acid:water (8:1:1 v/v). After drying the chromatogram, the layer was sprayed with the Lisboa reagent (0.5 ml anisaldehyde in 50 ml glacial acetic acid and 1 ml conc. sulfuric acid) and the spots were visualized by heating for 10 min at 100°C /4/. The sugar analysis showed the presence of glucose and rhamnose.

Identification of the anthocyanin

A sample of the anthocyanin obtained in the course of the isolation procedure was dissolved in MeOH and applied to a cellulose layer (Merck 5716), then developed in a mixture of acetic acid:hydrochloric acid:water (15:3:82 v/v) and in *n*-butyl alcohol:acetic acid:water (4:1:5 v/v, upper layer) /5/. The R_f values agreed with those of paper chromatography /6/ and coincided with the values of delphinidin-3-rutinoside-5-glucoside.

PARTIAL HYDROLYSIS

The anthocyanin was hydrolyzed for 1 h in 1 N HCl, at 100°C. Samples were examined in every 5 min on cellulose layer (Polygram Cel 300, Macherey Nagel), and were developed in a mixture of n-amyl alcohol:acetic acid:water (2:1:1 v/v). The partial hydrolysis resulted in five compounds, the hydrolysis products of delphinidin-3-rutinoside-5-glucoside.

ANALYSIS BY SPECTROPHOTOMETRY

After scraping off the original anthocyanin and its hydrolysis products from thin-layers, they were dissolved in 0.01% HCl in MeOH, and the spectra were obtained in the range 250--600 nm. Compared with the values given in the literature /7,8/ complete agreement was found.

SUMMARY

According to the present investigations, the anthocyanin of Consolida orientalis Schröd. is delphinidin-3-rutinoside-5-glucoside.

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CHARACTERISTIC FLAVONOIDS OF *LILIUM CANDIDUM* L. AND THEIR DISTRIBUTION IN DIFFERENT PLANT PARTS

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INTRODUCTION

In this paper we report new results on the phytochemical screening of Lilium candidum L.

As a result of a systematic survey of traditional popular medicines in Hungary, our interest has been drawn to white lily. Several data are reported about the medicinal uses of the different plant parts in various preparations as home remedies. In some regions of Transdanubia a home-made alcoholic extract is prepared from the freshly chopped perianth of Lilium candidum and used in curing inflammations of the eye accompanied by discharge and dermatitis.

Our team has been engaged in the study of the flavonoids of Lilium candidum and the presence of a new compound, lilyn (kaempferol 3-O-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside) has been reported [1].

Continuing our phytochemical screening of Lilium candidum L., kaempferol 3-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside (1) has been isolated and identified in the perianth, which has been not described previously in Lilium species.[x] Using the isolated compound and lilyn for comparison, the flavonoid composition in dried flower parts have been studied. In contrast whith the literature, this is dominated by kaempferol glycosides both in the perianth and stamens [3].

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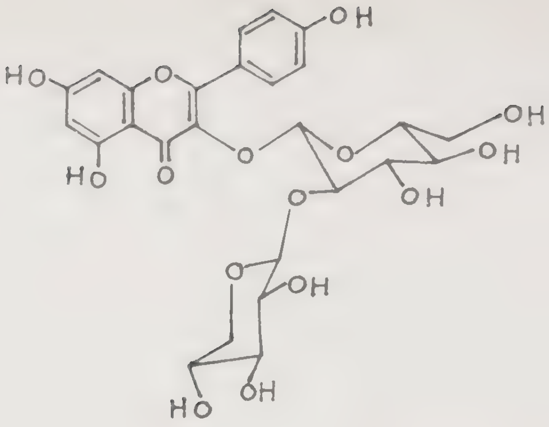
[x] V.M. Chari and H.Wagner [6] assigned structure (1) to natural leucoside.



(1)

RESULTS AND DISCUSSION

Using the multiplicities and chemical shifts in the ^{13}C-NMR spectrum, the lines are readily divided into two groups: the aglycon lines correspond to kaempferol substituted at C-3, while the remaining 11 lines are characteristic of a disaccharide. By detailed analysis of the chemical shifts as a pattern of substituted sugars, using data from the literature and our C-13 data bank on sugars, we concluded that the structure is a 2-substituted glucose where the substituent is β-D-xylose. The proton spectrum and the one-bond carbon-proton coupling constant ($^{1}\underline{J}_{CH}$) verify the beta anomeric state of both pyranoid rings ($^{3}\underline{J}_{H_1H_2}$ is axial-axial) and the same fact can also be deduced from the carbon shift of the anomeric carbons. The substitution pattern is well reflected in the α-and β-shift changes induced by the substitution at C-2" in the glucose ring. The NMR spectra support the structure (1) and exclude any other linkage possibility (Tables 1 and 2).

Lilyn and kaempferol 3-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside have been identified in the dried stamens too, using TLC and UV-VIS spectroscopy. The ratio of these compounds was studied in different flower parts (dried stamen and perianth) by direct TLC densitometry, Fig.1 and Table 3.

Table 1

Characteristic ^{1}H-NMR data of kaempferol 3-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside of structure (1)

	δ_{pp}^{TMS}	$^{3}J_{HH}^{Hz}$
H-1''	5.34 d	7.5
H-1'''	4.74 d	7.2
H-2'''	3.97 t	
H-2'	3.94 t	
H-6	6.45 s	
H-8	6.27 s	
H-3'	8.15 d	9.0 Hz
H-5'	6.98 d	
CD_3OD, room temperature		

The main flavonoid glycoside is lilyn in the perianth, and kaempferol 3-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside in the stamen.

EXPERIMENTAL

The dried and powdered perianths (100 g) were extracted with 2x500 ml of methanol at room temperature. The extracts were combined, and the solvent was removed under reduced pressure. The crude dried residue (10.5 g) was then extracted with 3x20 ml of hot methanol, filtered, and poured to a polyamide suspension swollen in water. After thorough shaking, the methanol was evaporated under reduced pressure. The suspension was collected on a filter paper, and it was washed with distilled water until the filtrate became colourless. The flavonoids were removed from the polyamide with methanol. The purified extract was concentrated to 2 ml under reduced pressure and subjected to column chromatography (polyamide, 40x1.5 cm). Elution was started with water, followed by methanol-water mixtures

Table 2

^{13}C-NMR chemical shifts (δ^{TMS}_{ppm}) and multiplicities (M) for kaempferol 3-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside of structure (1).(Taken at 25.16 MHz and room temperature.)

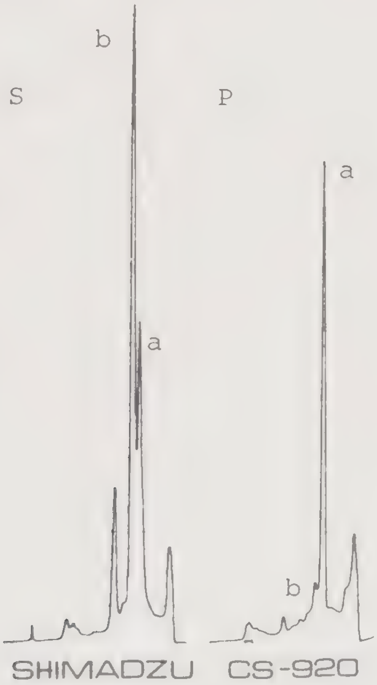
Carbon	δ	M	Me β-D-xylopyranoside [2]
2	158.4	1	
3	$\underline{134.9}$	1	
4	179.6	1	
5	162.9	1	
6	100.1	2	
7	166.5	1	
8	94.9	2	
9	158.2	1	
10	105.3	1	
1′	122.7	1	
2′	132.3	2	
3′	116.2	2	
4′	161.4	1	
5′	116.2	2	
6′	132.3	2	
1′′	101.3	2	
$^1J_{CH}$:	168 Hz		
2′′	$\underline{80.4}$	2	
3′′	76.8	2	
4′′	70.2	2	
5′′	75.0	2	
6′′	61.6	3	
1′′′	105.5	2	105.1
$^1J_{CH}$:	163 Hz		161 Hz
2′′′	75.1	2	74.0
3′′′	77.1	2	76.9
4′′′	71.0	2	70.4
5′′′	66.6	3	66.3
Reference:	CD_3OD (49.0 ppm)		
Solvent:	CD_3OD		

Chemical shifts belonging to substituted carbons are underlined

Table 3

Distribution of lilyn (a) and kaempferol 3-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside (b) in dried flower parts using TLC-densitometric method.

Plant part	Absolute value Perianth (a) and (b) =100%		Distribution (a) + (b) area=100%	
	(a)	(b)	(a)	(b)
Perianth (P)	100	100	80.96	19.04
Stamen (S)	119.26	663.95	43.30	56.70
Experimental data:	1.0 g powdered plant part in 3.0 ml methanol; TLC : see, Experimental			



254 nm; Sensitivity: 100 mV;
Paper speed: 2.5 cm/sec.

Fig.1

TLC densitogram of dried perianth (P) and stamen (S)
a. lilyn: b. kaempferol 3-O-β- D-xylopyranosyl-(1→2)-β-D-glucopyranoside

with the methanol content increasing gradually by 10 %, and finally finished with methanol. The eluent composition was changed at every 30 ml, the volume of fractions was 5 ml. In this way 32 mg of pure substance was obtained, which, according to its chromatographical behaviour, was assumed to be a diglycoside. Acid hydrolysis was carried out with 2 N HCl (one hour reaction time, in a sealed vial, at 100°C). After shaking and separating the reaction mixture with diethyl ether, the aglycon was identified as kaempferol by TLC and UV-VIS spectroscopy, comparing it with literature data and with an authentic sample [1]. The UV spectroscopic data of the isolated compound were: absorption maxima 268 nm, 286 nm sh; 300 nm, 308 nm sh; 350 nm sh. $[\alpha]_D = -52.5^\circ$ (methanol c=0,9)
The sugar part was identified in the neutralized acid phase by TLC on microcrystalline cellulose in acetone--isopropanol-0.1 M lactic acid (4:4:2); detection with aniline phthalate reagent (110°C, heating 1 min). Co-chromatography with authentic samples resulted in the identification of the sugar components of the isolated flavonoid as xylose (R_f = 0.54) and glucose (R_f = 0.38). The TLC data of the isolated compound were: Kieselgel G; ethyl acetate-methanol-water (77 :15:8); detection with $AlCl_3$, yellow: R_f = 0.49 (R_f of lilyn = 0.41).

The structure was elucidated by ^{1}H- and ^{13}C-NMR spectroscopy. The carbon-13 NMR spectrum was taken at 25.16 MHz and at room temperature in d_4-methanol (32 mg/ 6 ml). Chemical shifts were referenced to TMS(i) using the solvent line as a reference peak (49.0 ppm). Multiplicities were directly measured using the Attached Proton Test pulse sequence [5]. Proton NMR data were measured at 250, 200 and 100 MHz, respectively, in the same solution. Solvent suppression has been achieved by double quantum filtering [4] or by the inversion recovery method.

The authors are indebted to dr. G. Batta and I.Perczel for taking the high-field ^{1}H-NMR spectra.

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ISOLATION OF ANTHOCYANIDINS FROM NATURAL SOURCES

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Anthocyanin compounds frequently occur in the vegetable kingdom /1-4/. Flowers, fruits and other parts of plants contain these compounds which give different colours from orange-red to violet-blue. The tone of colour depends on the chemical character of the compounds; first of all it is decided by the number of hydroxyl- and methoxyl-groups attached to the aromatic rings. The tone shows a darkening character with the growing number of these groups.

Cyanidin (Fig. 1) derivatives, as a sub-group of the anthocyanins, are the most widespread in nature. They give the characteristic red colour of many plants.

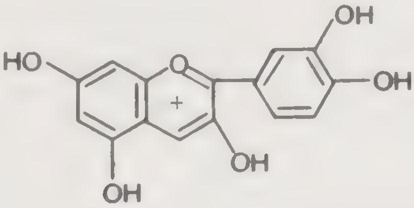

Fig. 1. Cyanidin (3, 5, 7, 3′, 4′ - pentahydroxyflavylium)

Cyanidin is almost always present in glycoside form in natural sources. In this way water-solubility is ensured. The sugars are connected to the hydroxyl groups at the three-or five-positions. Both of these hydroxyl groups are substituted by sugars in the diglucosides.

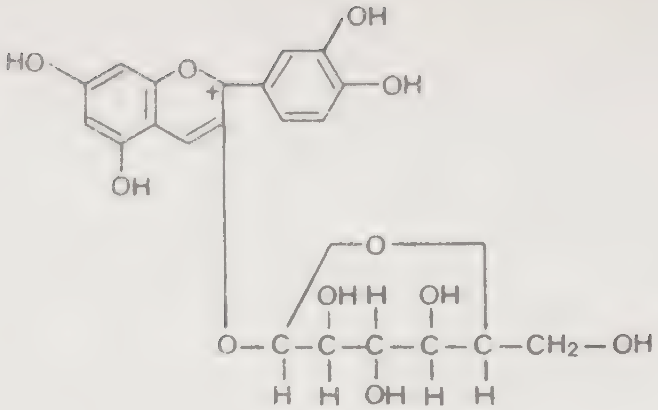

Fig. 2. Cy-3-monoglucoside (Chrysanthemin)

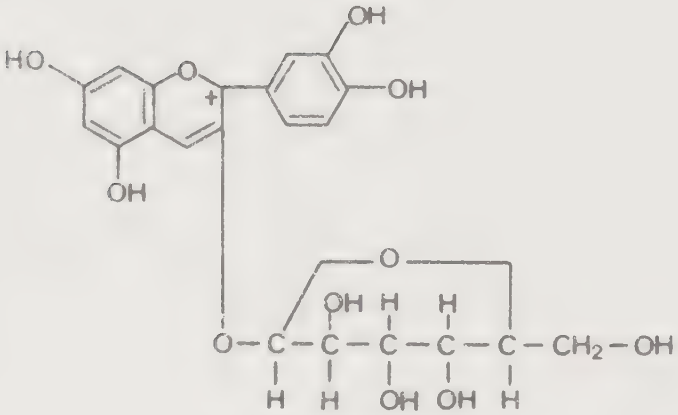

Fig. 3. Cy-3-monogalactoside (Idaein)

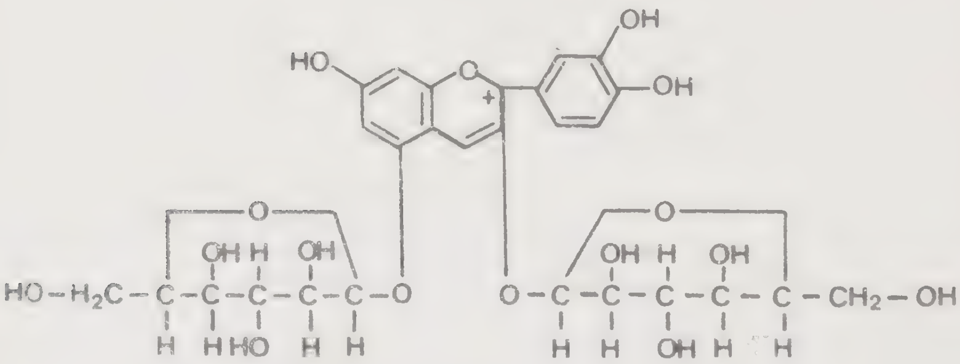
OH
HO
O
+
OH
O
OH H OH
HO – H₂C – C – C – C – C – C – O
H H HO H H
O
OH H OH
O – C – C – C – C – C – CH₂ – OH
H H OH H H

Fig. 4. Cy-3,5-diglucoside (Cyanin)

Well-known cyanidin glycosides are chrysanthemin, idaein and cyanin (Figs 2-4).

In the present work an example is given for the isolation, on preparative scale, of anthocyanidins from anthocyanin-containing natural sources.

EXPERIMENTAL

In our experiments we used raspberry (Rubus idaeus)as a natural source containing anthocyanins. In this fruit there are several cyanidin compounds, such as cyanidin-3-glucoside and cyanidin-3-sophoroside. In some species pelargonidin derivatives were also found, in a very small quantity /5/.

The fruit was pressed out and a six-fold volume of acetone was added to the juice. In this way the organic compounds with high molecular weight were precipitated from the solution and removed by filtration.

The ahthocyanins were adsorbed on the surface of basic lead acetate. This precipitate was washed with acetone, then the anthocyanins were eluted with dilute hydrochloric acid.

The solution containing the anthocyanidin glycosides was hydrolyzed at a temperature of 100 °C. The reaction mixture was then stored overnight at a temperature not higher than 4 °C, whereupon the cyanidin precipitated in crystalline form. This, still very crude, sample was several times recrystallized from HCl-containing methanol. The solvent was evaporated in each case in vacuum.

Purification was carried out by column chromatography. Two columns with different adsorbents were used.

The first separation was carried out on polyamide adsorbent, and the eluted sample was examined, after evaporation of the solvent, by paper chromatography on Whatmann No. 3 paper with two different solvent systems:

acetic acid: hydrochloric acid: water (30:3:10) and

n-butanol: acetic acid: water (11:14:25).

In both cases two spots were detected (Fig. 5).

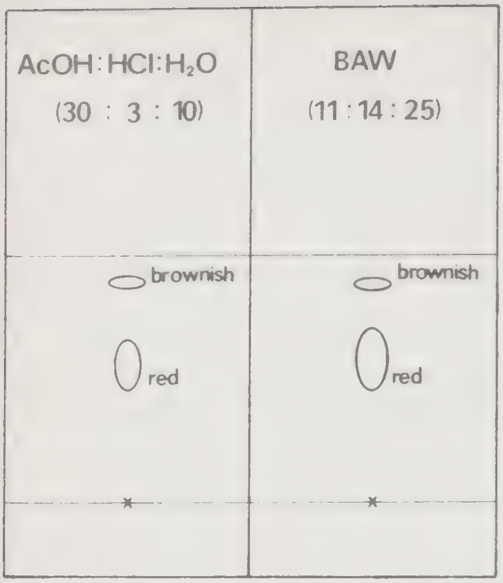

Fig. 5. Paper chromatography of crude cyanidin

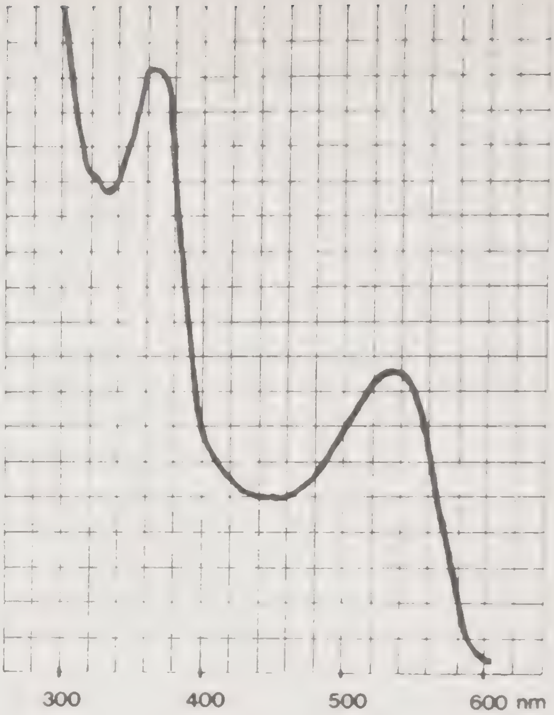

Fig. 6. Spectrum of crude cyanidin.HCl (Pye Unicam SP-8-100)

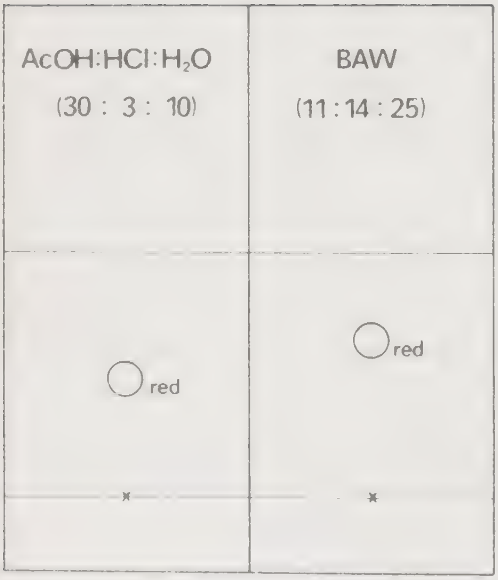

Fig. 7. Paper chromatography of pure cyanidin

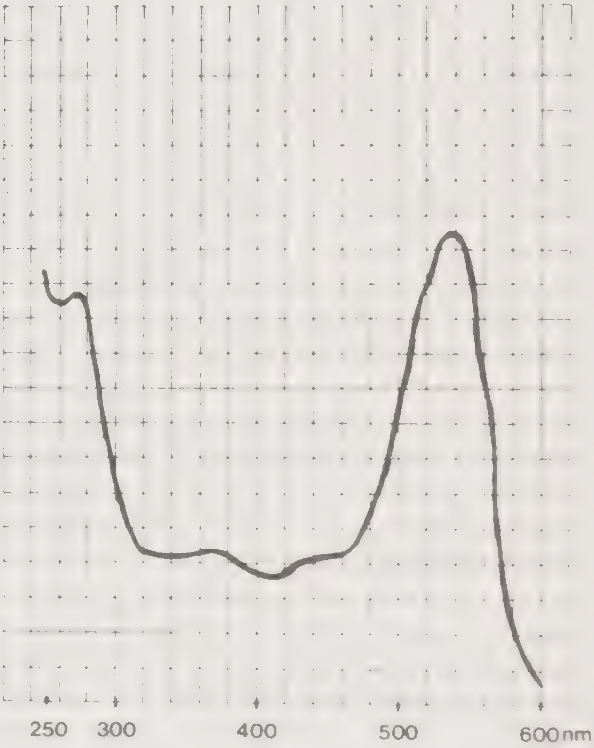

Fig. 8. Spectrum of pure cyanidin.HCl (Pye Unicam SP-8-100)

The VIS-UV spectrum of this sample (Fig. 6) in a solution of HCl-containing methanol showed two maxima in the visible region.

Further purification was carried out with the aid of Sephadex LH 20 adsorbent. In this way a pure compound was isolated. In paper chromatography it gave one spot under the same circumstances as earlier (Fig. 7).

The VIS-UV spectrum of the sample in a solution of 0.01 % HCl-containing methanol showed an absorption maximum at a wavelength of 535 nm, confirming the presence of cyanidin.

The infrared absorption spectrum could also be identified as that of cyanidin.

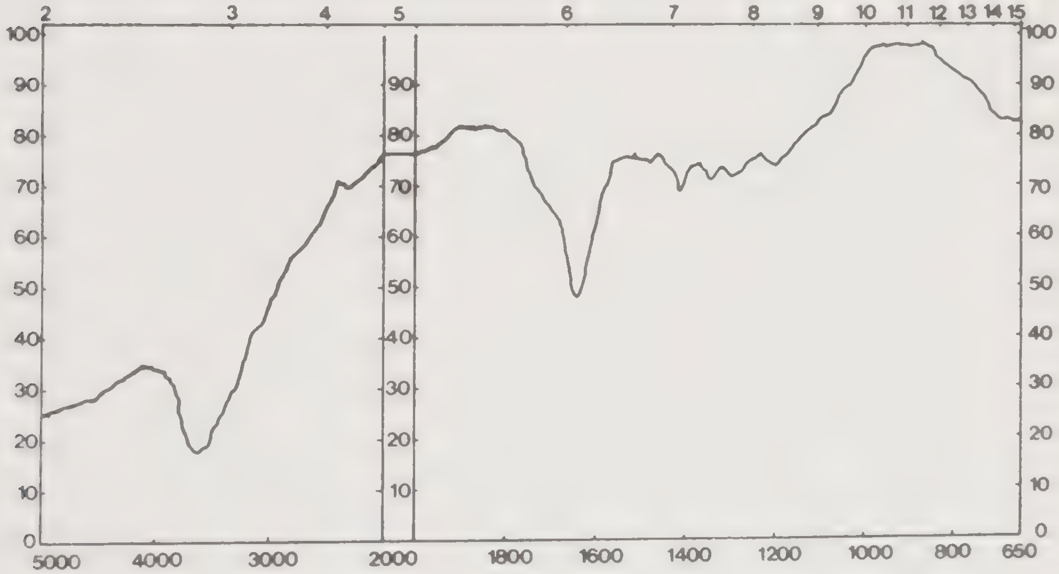

Fig. 9. IR spectrum of cyanidin.HCl

SUMMARY

As a result of these investigations a simple method is described for the isolation of pure cyanidin in preparative quantities from cyanidin glycoside-containing natural sources.

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CHARACTERISTIC CONSTITUENTS OF THE CHERRY PEDUNCLE EXTRACT NOVICARDIN: HPLC FINGERPRINT

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The diuretic effect of aqueous extracts of cherry peduncle has been known in folk medicine for a long time /1/. Some handbooks mention the antiorrhorrheic effect of the cherry stalk and note that the peduncle is an essential component of slimming cure teas /2/. Its mucolytic action and very good influence on the heart are mentioned as well /3/. The positive inotropic effect of the extract with organic solvents of cherry stalk was verified by pharmacological studies /4/. The preparation of such an active extract, under the name Novicardin, was protected by several patents, e.g. /5/.

A survey of the literature shows that a number of data are available about the components of cherries (Prunus avium and Prunus cerasus). Both plants contain organic acids, amino acids, terpene derivatives and phenolic compounds /6/. Many flavonoids have been isolated from the bark, heartwood, leaves, fruit, and peduncle. In the peduncle Theodossiou /7/ identified the glycosides of dihydrowogonin, naringenin, taxifolin, aromadendrin, and quercetin by chromatography. Wagner et al. /8/ isolated dihydrowogonin-7-O-glycoside, Fodor et al. /9/ identified naringenin, sakuranetin, chrysin, and dihydrowogonin by HPLC.

Fodor et al. have developed a HPLC method to detect the components of extract of cherry stalk (Novicardin) /9/. Our team cooperated with them to isolate and characterize the compounds which can be detected by HPLC. We report here on the flavonoids which have been isolated from Novicardin so far.

By means of this HPLC method a number of flavonoids can be detected (see Fig. 1). About eleven of them are present in

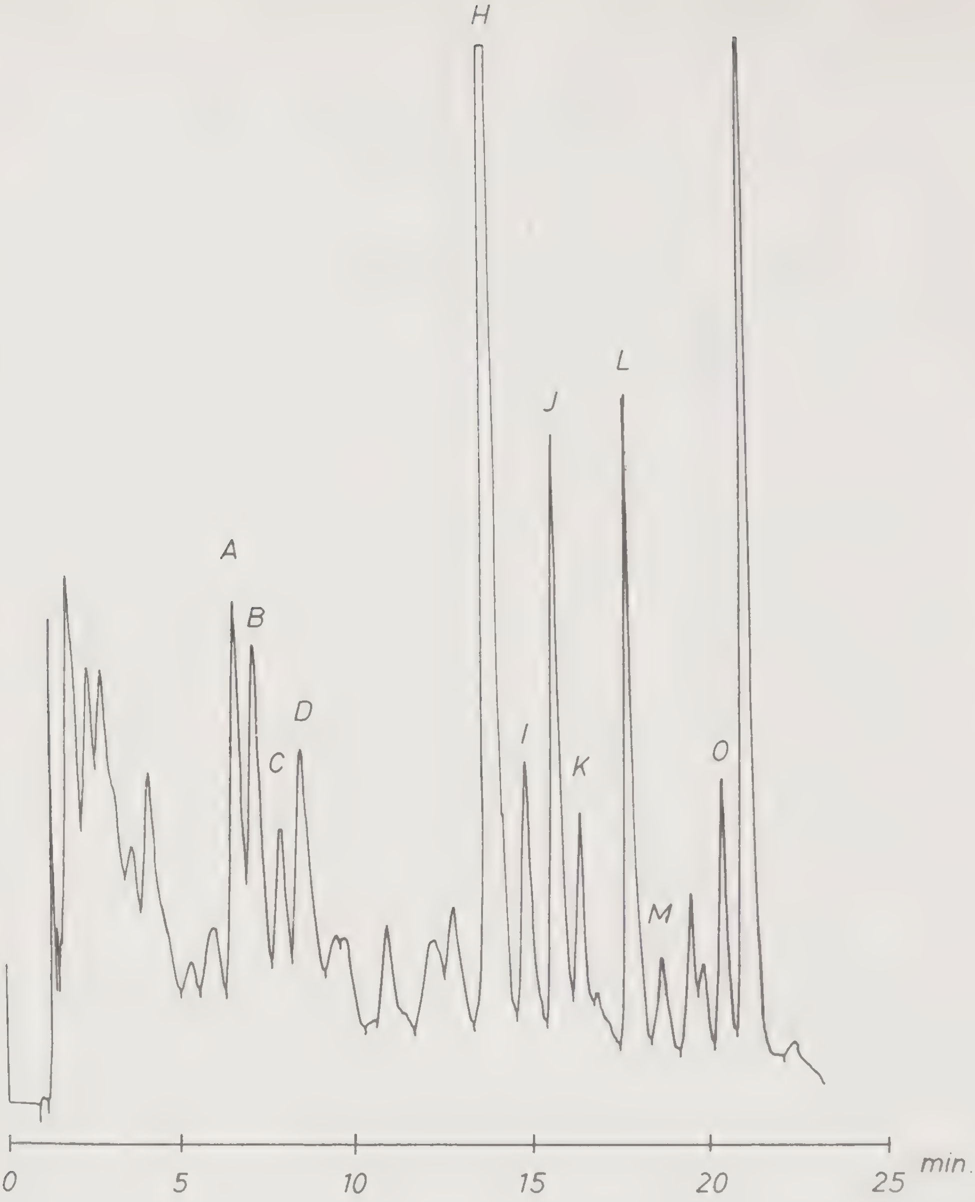


Fig. 1. Separation of Novicardin on Knauer RP-8 5 µ coloumn
Solvents: primary 80% 0.01 m H_3PO_4, 20% MeOH
secondary MeOH. Flow rate: 1.5 ml/min.
Pressure: $9.8 \cdot 10^7$ Pa. Detection: 280 nm

larger amounts; we attempted to isolate them by column chromatography.

Novicardin was fractionated on a polyamide column using different mixtures of ethyl acetate-methanol-water. Thus we obtained six rough fractions, which were separated further into individual components on Sephadex LH20 gel.

The pure compounds were designated with letters of the alphabet. The structures of the compounds were deduced from their spectral characteristics (UV, NMR, MS). Identity was confirmed in each case by direct comparison with an authentic sample. In the case of glycosides, after hydrolysis, the sugars were identified by paper chromatography.

In this way, sixteen compounds have been isolated and identified as follows:

Compound A (m.p. 238-242°C) was identified as dihydromorin (Ia) by the following spectral data: UV_{max}^{MeOH} 293, 342 nm, + NaOMe 330 nm, + NaOAc 330 nm, + $AlCl_3$ and $AlCl_3$/HCl 318, 398 nm. MS (*m/z*) (rel.int., %): 304 (23), 275 (42), 164 (18), 153 (100), 152 (36). NMR ($CDCl_3$): 4.5 (1H, q, H-3), 5.0 (1H, d, H-2), 5.75 (1H, d, C-3 OH), 5.93 (2H, dd, H-6, H-8), 6.73 (2H, s, H-5, H-6), 6.87 (1H, s, H-8).

Compound B (m.p. 166-169°C) was characterized as salipurposide (Ib): UV_{max}^{MeOH} 286 nm, + NaOMe 252 (sh), 329 nm, + NaOAc 252, (sh) 329 nm, + $AlCl_3$ and $AlCl_3$/HCl 286 nm. On hydrolysis, the aglycone showed bathochromic shift with $AlCl_3$, which did not change with $AlCl_3$/HCl. The aglycone was identified as naringenin by TLC, and the sugar as glucose by PC.

Compound C (m.p. 268-271°C) proved to be genistin (IIIa): UV_{max}^{MeOH} 264, 332 (sh), + NaOMe 272, 370 (sh) nm, + NaOAc 264, 332 (sh) nm, + $AlCl_3$ and $AlCl_3$/HCl 274, 385 nm. After hydrolysis, the resulting aglycone was identical with compound J (genistein), the sugar being glucose.

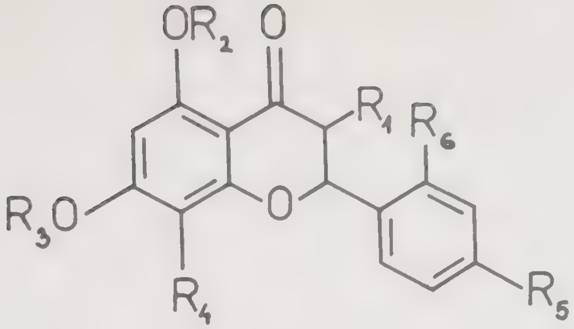
Compound D (m.p. 228-230°C) was identified as prunin (Ic): UV_{max}^{MeOH} 285, 339 (sh) nm, + NaOMe 288, 408 nm, + NaOAc 286, 337 (sh) nm, + $AlCl_3$ and $AlCl_3$/HCl 310, 384 nm. After hydrolysis, the aglycone showed a bathochromic shift of 39 nm with NaOAc. The aglycone was identified as naringenin; the sugar proved to be glucose.

Compound H (m.p. 226-229°C) was characterized as dihydrowogonin-7-O-glucoside (Id): UV_{max}^{MeOH} 289, 350 nm, + NaOMe 289, 371 nm, + NaOAc 289, 368 nm, + $AlCl_3$ and $AlCl_3$/HCl 311, 405 nm. On hydrolysis it gave compound L (dihydrowogonin) and glucose. The aglycone showed a bathochromic shift of 37 nm, due to the free OH group at position 7.

Compound I (m.p. 249-253°C) was found to be naringenin (Ie) based on the following spectral data: UV_{max}^{MeOH} 289, 345 (sh) nm, + NaOMe 247 (sh), 326 nm, + NaOAc 250 (sh), 280 (sh), 329 nm, + $AlCl_3$ and $AlCl_3$/HCl 313, 380 nm. MS (m/z) (rel.int., %): 272 (78), 153 (100), 152 (25). The identity was confirmed by direct comparison with an authentic sample.

Compound J (m.p. 304-308°C) was identified as genistein (IIIb): UV_{max}^{MeOH} 262, 293 (sh), 334 (sh) nm, + NaOMe 276, 325 (sh), + NaOAc 276, 330 nm, + $AlCl_3$ and $AlCl_3$/HCl 276, 310 (sh), 380 nm. MS (m/z) (rel. int., %): 270 (100), 269 (22), 153 (58), 152 (37). NMR ($CDCl_3$): 6.5 (1H, d, H-6), 6.75 (1H, d, H-8), 6.85 (2H, d, H-3', H-5'), 7.41 (2H, d, H-2', H-6'), 8.4 (1H, s, H-2).

Compound K (m.p. 244-247°C) proved to be chrysin-7-O-glucoside (IIa): UV_{max}^{MeOH} 272, 331 (sh) nm, + NaOMe 282, 385 nm, + NaOAc 276 nm, + $AlCl_3$ and $AlCl_3$/HCl 287, 325 (sh), 399 (sh) nm. After hydrolysis, the aglycone showed a bathochromic shift in the presence of NaOMe. The aglycone was identical with compound O (chrysin) and the sugar was glucose.



I

Ia: R_1=R_5=R_6=OH; R_2=R_3=R_4=H

Ib: R_1=R_3=R_4=R_6=H; R_5=OH; R_2=gl

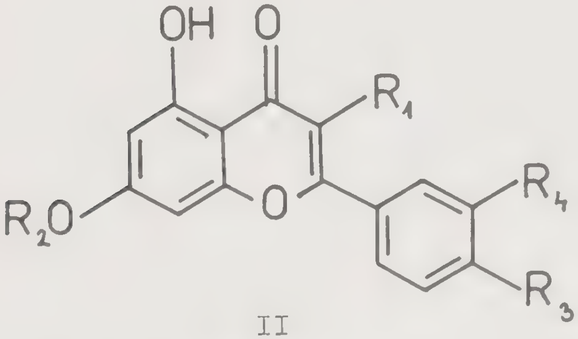
Ic: R_1=R_2=R_4=R_6=H; R_5=OH; R_3=gl

Id: R_1=R_2=R_5=R_6=H; R_4=OCH_3; R_3=gl

Ie: R_1=R_2=R_3=R_4=R_6=H; R_5=OH

If: R_1=R_2=R_3=R_5=R_6=H; R_4=OCH_3

Ig: R_1=R_2=R_4=R_6=H; R_5=OH; R_3= CH_3



II

IIa: R_1=R_3=R_4=H; R_2=gl

IIb: R_1=R_2=R_3=R_4=H

IIc: R_1=O-rutinose; R_2=H; R_3=R_4=OH

IId: R_1=R_3=R_4=OH; R_2=H

IIe: R_1=OH; R_2=R_3=R_4=H

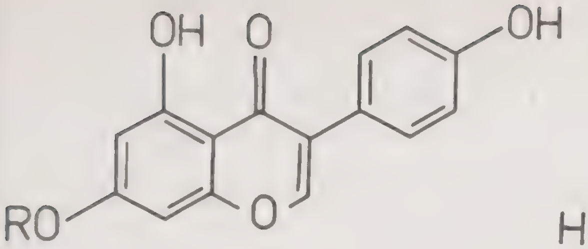
Compound L (m.p. 148-150°C) was identified as dihydrowogonin (If) from its spectral data: UV_{max}^{MeOH} 294, 340 (sh) nm, + NaOMe 253 (sh), 333, 341 (sh) nm, + NaOAc 332 nm, + $AlCl_3$ and $AlCl_3$/HCl 316, 401 nm. MS (m/z) (rel. int., %): 286 (100), 271 (10), 182 (77), 167 (90), 153 (20). NMR ($CDCl_3$δ): 3.00 (2H, m, H-3), 3.9 (3H, s, OCH_3), 5.5 (1H, dd, H-2), 6.15 (1H, s, H-6), 7.55 (5H, s, B-ring protons).

Compound M (m.p. 125-128°C) was sakuranetin (Ig) one the basis of the following spectral data: UV_{max}^{MeOH} 290, 333 (sh) nm, + NaOMe 290, 368 nm, + NaOAc 290, 333 (sh) nm, + $AlCl_3$ and $AlCl_3$/HCl 312, 380 nm. MS (m/z) (rel. int., %): 286 (60), 193 (25), 167 (75), 166 (22), 119 (35). NMR ($CDCl_3$δ): 2.75-3.2 (2H, br m, H-3), 3.8 (3H, s, OCH_3), 5.35 (1H, dd, H-2), 6.05 (1H, s, H-6), 6.10 (1H, s, H-8), 6.8 (2H, d, H-3′, H-5′), 7.35 (2H, d, H-2′, H-6′). Its identity was confirmed by comparison with an authentic sample.

Compound O (m.p. 288-291°C) was characterized as chrysin (IIb) from its spectral data: UV_{max}^{MeOH} 270, 315 (sh) nm, + NaOMe 278, 368 nm, + NaOAc 278, 368 nm, + $AlCl_3$ and $AlCl_3$/HCl 284, 335, 395 nm. MS (m/z) (rel. int., %): 254 (100), 253 (20), 152 (25), 125 (35). The identity was confirmed by direct comparison with an authentic sample.

Compounds E, F, G, P, and Q, respectively, were identified as rutin (IIc), quercetin (IId), galangin (IIe), catechin (IV), and epicatechin (V) by their reactions and chromatographic comparison with authentic samples.

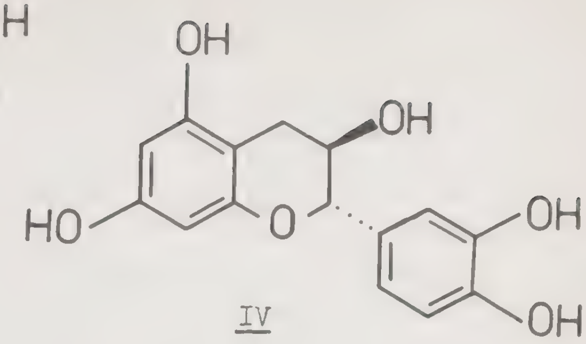
Summing up the results: our purpose was to isolate and characterize the main compounds in Novicardin, the extract of cherry stalk. Sixteen compounds have been isolated so far. They were identified by their spectral data and/or by direct comparison with authentic samples. All these flavonoids are known compounds. Four of them, dihydromorin (Ia), and salipurposide



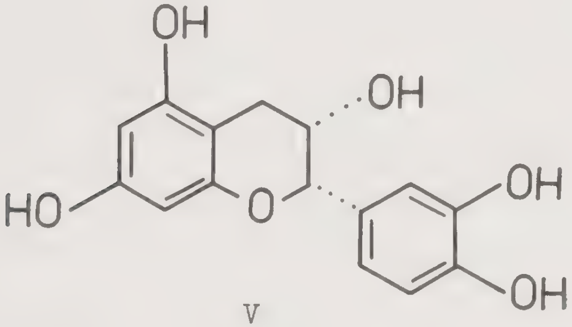
III

IIIa: R=gl

IIIb: R=H



IV



V

(Ib), chrysin-7-O-glycoside (IIa), galangin (IIe) have not been isolated before from this plant.

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PHARMACOLOGICAL PROPERTIES OF A NOVEL FLAVAN DERIVATIVE ISOLATED FROM THE NEW ZEALAND CONIFER *PHYLLOCLADUS ALPINUS*: CARDIOVASCULAR ACTIVITIES OF PHYLLOFLAVAN IN RATS

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INTRODUCTION

While surveying the New Zealand conifers chemotaxonomy, we analyzed the flavonoid /1/ and flavanoid /2/ composition of several specimens belonging to the genus *Phyllocladus*.

A remarkable peculiarity of this genus is that the functions of their leaves are carried out by flattened and fused branchlets called phylloclades /3/. The genus *Phyllocladus* comprises five species of evergreen trees. Three are endemic to New Zealand, *P. alpinus*, *P. trichomanoides* and *P. glaucus*; one is endemic to Tasmania, *P. aspleniifolius*, and one is ranging from the Philippines to New Guinea, *P. hypophyllus* /4/.

The ethyl-acetate extracts of the phylloclades when examined by 2D-TLC or 2D-PC and visualized selectively with vanillin-HCl reagent, revealed that three major flavans are ubiquitous in the genus : catechin, epicatechin and a novel constituent which we named "phylloflavan" /2/.

In the sample of *P. alpinus*, from which we isolated phylloflavan, this new flavanoid constitutes about twice the combined bulk of catechin and epicatechin, the latter two flavans being present at about the same level (fig. 1).

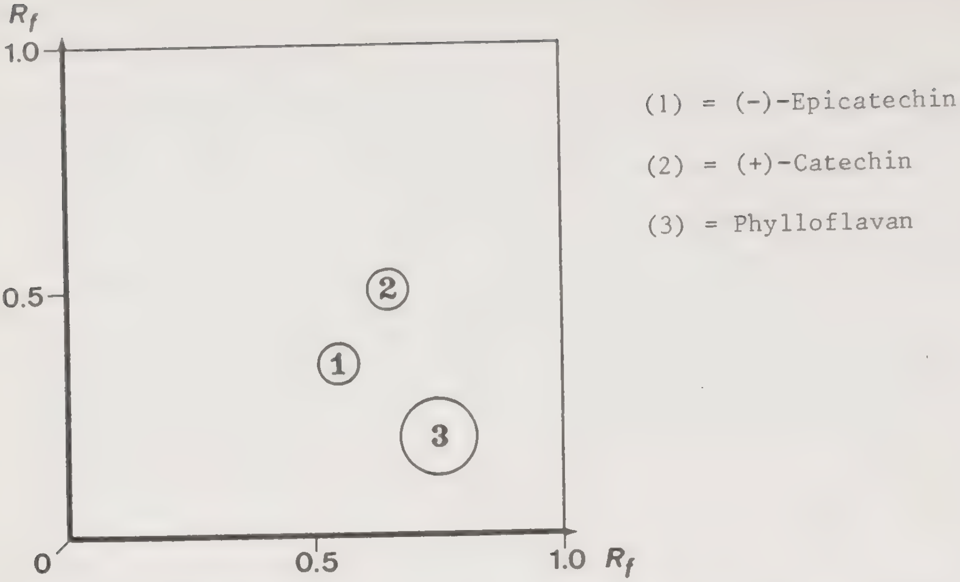

Fig. 1 : 2D-TLC on cellulose (Schleicher & Schull plates) of the EtOAc extract from *Phyllocladus alpinus*.

The quantitative separation of phylloflavan was readily effected by chromatography on Sephadex LH-20 and its structural formula was proved to be *ent*-epicatechin-3-δ(3,4-dihydroxyphenyl)-β-hydroxypentanoate /2/ (fig. 2).

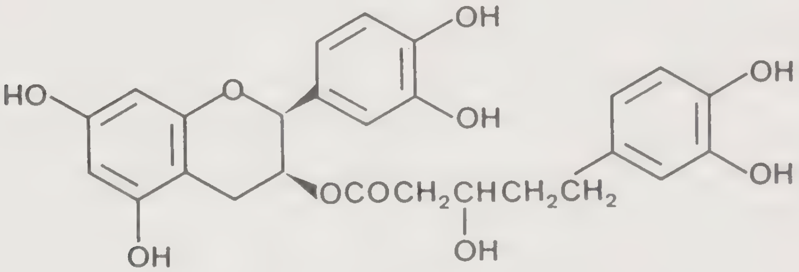

Fig. 2 : Structure of phylloflavan

It must be emphasized that, while catechin and epicatechin found in *P. alpinus* are of the normal type with *2R* configurations, the flavan moiety in phylloflavan is of the opposite *2S* configuration, which is unusual.

As we had previously investigated the physiological activities of some other flavanols and flavonoids, we found interesting to submit phylloflavan to a similar series of assays.

The available amount of phylloflavan (abbreviated here as PF) did not allow a thorough study of its pharmacological properties. We have limited our aim to a first examination of its cardiovascular activities in rats, some of these requiring to be confirmed further.

A. Cardiovascular system

1. Fast intravenous injection

From 1 $mg.kg^{-1}$ - dose threshold - PF causes in rats anaesthetized by pentobarbital sodium a rise of the systemic arterial pressure, which is proportional to the injected dose up to 50 $mg.kg^{-1}$. The hypertension appears and disappears slowly, within 2 to 5 minutes. Above 50 $mg.kg^{-1}$, an hypotension appears immediatly which evolves quickly before hypertension takes place. The vascular response is then biphasic. This is reproducible (fig. 3).

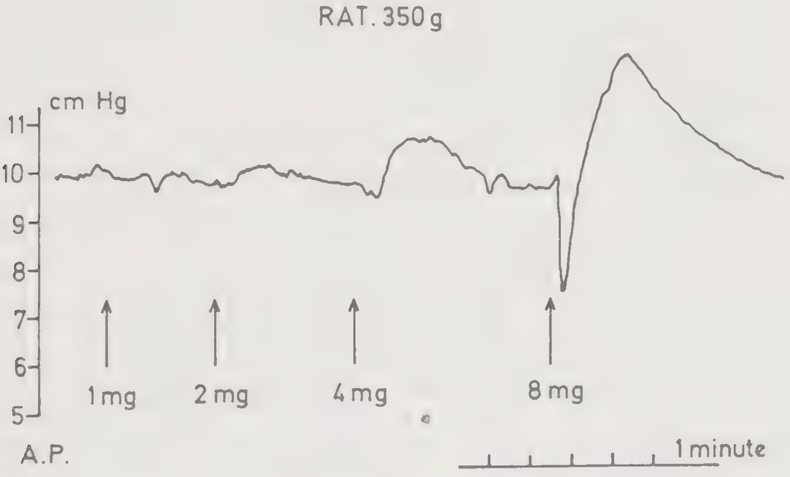

Fig. 3 : Systemic arterial pressure of the rat :
- Y axis : arterial pressure (cm-Hg)
- X axis : time (min)
- At each arrow : intravenous injection of phylloflavan : 1, 2, 4 and 8 mg, respectively.

2. Continuous intravenous injection

From a solution at 10 $mg.ml^{-1}$, the continuous perfusion of 3-4 $mg.min^{-1}$ of PF, up to 60 to 80 $mg.kg^{-1}$ as a whole, causes a progressive rise of the arterial pressure, whithout any intercurrent hypotension.

The hypertension disappears slowly as soon as the perfusion is stopped. This can be brought on again when the same perfusion is renewed (fig. 4).

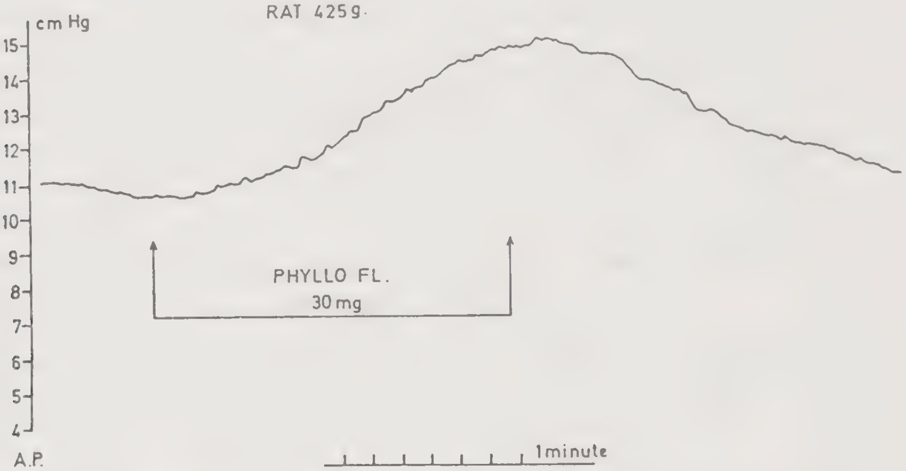

Fig. 4 : Systemic arterial pressure of the rat, as in fig. 3.
- Between arrows : perfusion of a solution of phylloflavan at 10 mg.ml^{-1}. As a whole, 30 mg were injected.

3. Attempted interpretation

3.1. The most apparent cardiovascular manifestation is expressed by the systemic arterial hypertension. This has been analyzed in relation with adrenergic mechanisms. Hypertension retains the same quantitative and qualitative characteristics in adrenalectomized rats and in the guanethidine-treated animals (20 mg.kg^{-1}), which can no longer release endogenous catecholamines, neither by the adrenal glands, nor by the orthosympathetic post-ganglionic terminals. The hypertension appears to be less important after phentolamine (20 mg.kg^{-1}) : the blockade of the α-adrenergic receptors attenuates but does not suppress it.

So, PF does not behave like a direct sympathomimetic amine. However, it should be able to attach to the α-adrenergic receptors.

This point needs to be checked in other animals species.

In other respects, the hypertensive effects of noradrenaline (0.1 to 0.4 µg) are not modified by PF : there is no potentiation.

3.2. What is the nature of the initial arterial hypotension which is observed after fast injection of doses higher than 50 $mg.kg^{-1}$?

It does not depend on a release of mast cells amines (histamine and 5-hydroxytryptamine), as induced by rutosides, for example. Even at high doses, PF does not cause any oedema of the extremities, which is the usual sign of such a release.

This hypotension does neither depend on a production of kinins. After intravenous perfusion of 100 mg PF, the injection of 150 mg ellagic acid - a kinino-releasing agent active in rats - still causes a drop of the arterial pressure : the plasma-kininogens are thus left intact after administration of PF. Conversely, in the rat which is first pretreated with ellagic acid (150 $mg.kg^{-1}$, iv), PF still remains hypotensive although the kininogens were exhausted.

PF does neither potentiate the prostaglandino-releasing power of bradykinin. The perfusion of PF at a rate of 60 $mg.kg^{-1}$ for 6 min. prolongs and reduces the post-bradykinin hypotension in the same way as the introduction of the same volume of NaCl 0.9%. In some rats however, the prostaglandino-releasing power seems to be slightly diminished.

B. Vascular permeability

When injected intraperitoneally in awakened rat at the dose of 100 $mg.kg^{-1}$, PF does not exert any inhibition on the development of the hind paw oedema locally induced by carrageenin (1 mg in one of the hind paws) within the 4 next hours. These observations must be confirmed with other proinflammatory agents.

C. Tolerance

Rats have well tolerated up to 200 mg PF, injected intravenously in discontinuous doses, during 2 or 3 hours.

No sign of general toxicity did appear. The same was observed in the rats which received 100 $mg.kg^{-1}$ intraperitoneally.

D. Comparison with other catechin derivatives and with other flavonoids

1. Catechin and epicatechin injected intravenously do not modify the arterial pressure of the rat in doses 5 to 6 times higher than that of phylloflavan /5/.

2. Catechin and epicatechin injected intraperitoneally exert an anti-oedematous activity which is likely connected to non-specific vascular lesions with local exudation /6/.

3. The dimeric procyanidins activate plasma-kininogens in a way similar to that of ellagic acid /5/.

4. The hydrosoluble rutin derivatives, as the hydroxyethyl rutosides (HER) among others, are amino-releasing substances from mast cells /7/.

E. Conclusion

By its hypertensive activity, phylloflavan is clearly distinguished from the other flavonoid derivatives which were investigated so far in the rat.

This new flavanoid possesses an extra catechol ring at the end of an alkyl side chain, which is obviously similar to catecholamines. Therefore, the proposed adrenergic mechanism of action of phylloflavan could be connected with a structure-activity relationship unique amongst bioflavonoids.

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PHARMACOLOGICAL PROPERTIES OF *MYRTILLUS* ANTHOCYANOSIDES: CORRELATION WITH RESULTS OF TREATMENT OF DIABETIC MICROANGIOPATHY

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INTRODUCTION

Modifications occur in several components of connective tissue, inclucluding capillary basal laminae and arterial walls, during the course of diabetes. A study was conducted to determine mechanisms responsible for certain results obtained with Vaccinium myrtillus anthocyanosides (VMA) on various aspects of diabetic microangiopathy /1/.

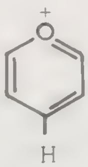
The chemical structure of anthocyanosides differs slightly from that of flavonoids (3): they are derivatives of phenyl-2-benzopyrylium (2); pyryllium cation is an oxonium in which the tetravalent oxygen atom has a positive charge (1).

In the case of pyrylium, its aromatic character due to the presence of conjugated double bonds is responsible for its stability.

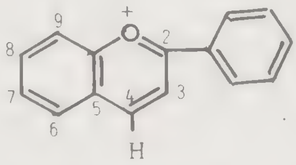
In a conjugated system such as the flavylium ion, the positive charge is not localized on a well-defined atom, but is distributed over the different atoms of the molecule. The true structure corresponds to a resonance hybrid between different limit formulae.

According to the position of heterosides fixed on the nucleus, certain properties may differ as a function of the various types of anthocyanosides.

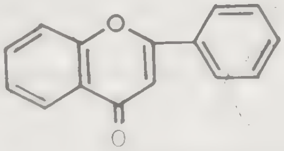
For this study we used VMA, its heterosides being distributed equally between positions 3 or 5.



Pyrylium (1)



Phenyl-2-benzopyrylium (2)
(Flavylium, oxonium form)



Flavone (3)

It is well established that diabetic microangiopathy is characterized histologically by thickening of the capillary basal laminae, with accumulation of collagen and of structural glycoproteins.

It is also known that among the factors active in the evolution of simple atherosclerosis there is early degradation of arterial elastin, as well as an increase in the types of collagen termed "polymeric" or again "insoluble", greater than that of the "soluble" types.

It has been clearly demonstrated /2/ that in diabetes these two alterations occur in connective tissue, associated at an early stage with the diabetic vasculopathy.

After assessing behaviour of connective tissue of the skin and gums of human diabetics, we conducted an experimental study of this behaviour on microvessels extracted from normal young and old rat brains and also from young rats made diabetic by streptozotocin.

Constant verification was conducted using conventional tests to ensure that the animals had really become diabetic.

Cerebral tissue was used because extraction and purification of microvessels is simpler in this region than in other tissues.

The results of the assays performed were as follows:

- 1) The total protein content of the capillaries of the older animals was higher by 20% than that in normal younger animals.

After four weeks of diabetes, total protein content in young diabetic animals had become equal to that of normal older animals.

- 2) Total collagen in normal older animals was higher by 50% than that in normal young animals. After four weeks of diabetes, collagen content in young diabetic animals had become equal to that of normal older animals (Fig. 2).

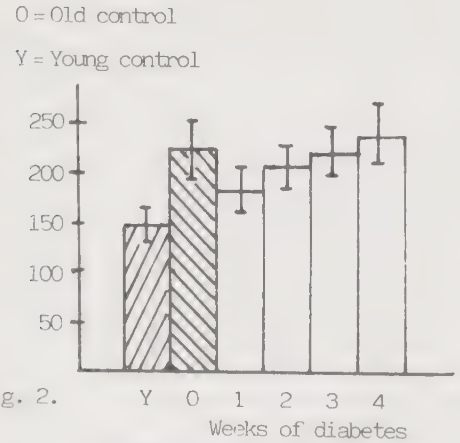


Fig. 2. Collagen content of the brain microvessels of normal and diabetic rats.

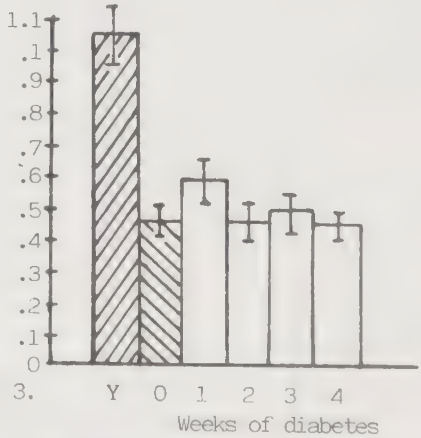


Fig. 3. Collagen of brain microvessels of normal and diabetic rats. Ratio of buffer-soluble to collagenase-soluble and residual collagen.

- 3) The ratio soluble collagen/insoluble collagen was greatly diminished in normal older animals when compared with normal younger animals; this indicates a strong increase in "polymeric" collagen during ageing.

After four weeks of diabetes, young animals were found to have the same values for the ratio soluble collagen/insoluble collagen as normal older animals (Fig. 3).

This increase in insoluble collagen during diabetes has been demonstrated clearly by electron microscopy; in thickened basal laminae of human diabetic capillaries one can clearly see numerous fibres of polymeric collagen never found in normal basal laminae.

INSULIN, ANTHOCYANOSIDES AND PROTEIN BIOSYNTHESIS

It is well established that the organism includes numerous cellular systems possessing other functions than those for which they seem to be specialized. An example is smooth muscle cells in arteries, since independently of their motor function they synthetize a basal lamina and also proteolytic enzymes such as elastase. It has also been demonstrated that elastolytic activity is present in human aorta and that this increases with age and with atherosclerosis /3/.

We therefore used cultures of smooth muscle cells from aorta to study cellular biosynthetic activities in experimentally-provoked diabetes in rats, when compared with cells of normal rats.

Assays were conducted on cells after several "passages" in culture using the conventional procedure. Quantitative evaluation of biosynthesis of proteins was made by adding labelled amino acids to the culture medium and measuring, after a given time T, the radioactivity acquired by the cells in which the amino acids had been incorporated.

Table 1

Estimation of total protein and collagen synthesis of ^{14}C - proline incorporation in extracellular matrix macromolecules of rat aorta smooth muscle cells. Effects of Anthocyanosides.

CELLS	RADIOACTIVITY IN TOTAL PROTEINS, CPM/μg DNA.10^{-3}	RADIOACTIVITY IN COLLAGEN (HYPRO) CPM/μg DNA 10^{-3}
NORMAL	21.2 ± 8.1	7.8 ± 4.5
NORMAL + ANTHO. 2 μg/ml	15.8 ± 9.3	3.9 ± 1.8
NORMAL + ANTHO. 20 μg/ml	6.8 ± 0.9 *	1.3 ± 5.2 *
DIABETIC	58.9 - 27.2**	15.3 - 6.9**
DIABETIC+ ANTHO 2 μg/ml	42.3 ± 18.3	10.2 ± 3.5
DIABETIC + ANTHO 20 μg/ml	12.3 - 1.58 *	5.2 - 5.3 *

* $P < 0.05$ ** $P < 0.01$

Results of this measurement demonstrated that the biosynthesis of proteins had more than doubled.

Note in this respect that after several passages in culture, the cells 'remember' having belonged to a host that had been made diabetic.

To continue the experiment, if one adds to the culture medium some VMA at a concentration of 2 µg/ml, one finds that the proteins synthetized have diminished by 23% in the controls and by 28% in the diabetics. With VMA at 20 µg/ml, proteins of controls were diminished by 66% and those of diabetics by 79% (Table 1).

With respect to collagen, there are reductions in biosynthesis of the same order as those of proteins.

Finally, comparison between the action of insulin and that of VMA under the same conditions demonstrates that insulin reduces by 22% the amount of collagen in diabetics, that the VMA concentration of 2 µg/ml reduces it by 58% and that an association of the two (insulin plus VMA) reduces it by 52% (Table 2).

Table 2

Collagen content expressed as µg of hydroxyproline by flask

Controls	0.094 ± 0.022	Diabetics	0.440 ± 0.060*
Controls + Ins	0.082 ± 0.017	Diabetics + Ins.	0.340 ± 0.081*
Controls + AVM	0.040 ± 0.030	Diabetics + AVM	0.182 ± 0.056**
Controls + I+AVM	0.063 ± 0.017	Diabetics + I+AVM	0.210 ± 0.042**

N = 8 flasks in each group. I : Insulin 100 µg/ml.

* P versus controls < 0,05 A : Anthocyanosides 2 µg/ml

ANTHOCYANOSIDES, INSULIN AND ELASTOLYTIC ACTIVITY

Smooth muscle cells that were studied to test the biosynthesis of certain proteins, also produce other proteins and proteases, and participate, with many other cells of the organism, in tissue turnover.

The elastolytic activity is dependent upon (with other factors related to the proteolysis itself) basic factors: biosynthesis of protease, an intracellular phenomenon, and transfer towards the extracellular compartment of this protease.

We conducted separate studies on the two phenomena by measuring degradation with elastase of the substrate n-succinyl-trialanine p-nitroanilide.

Biosynthesis of elastase was evaluated from cells isolated from their culture medium, and transfer was assessed in the culture medium. Elastolytic activity (O on the graph of Figure 4) is dependent upon the quantity of elastase present.

Columns in Fig. 4 list the results of this study, in which we compared the corrective actions of insulin (I) and anthocyanosides (A). It can be seen that biosynthesis and transfer of elastase were weak in normal control (C) and only slightly modified by insulin and VMA.

In diabetics, elastase is greatly increased in the cells and in culture medium, but insulin normalizes biosynthesis and transfer. Anthocyanosides are less active against biosynthesis but they reduce transfer by 70%, which appears to result from an action on the cell membrane.

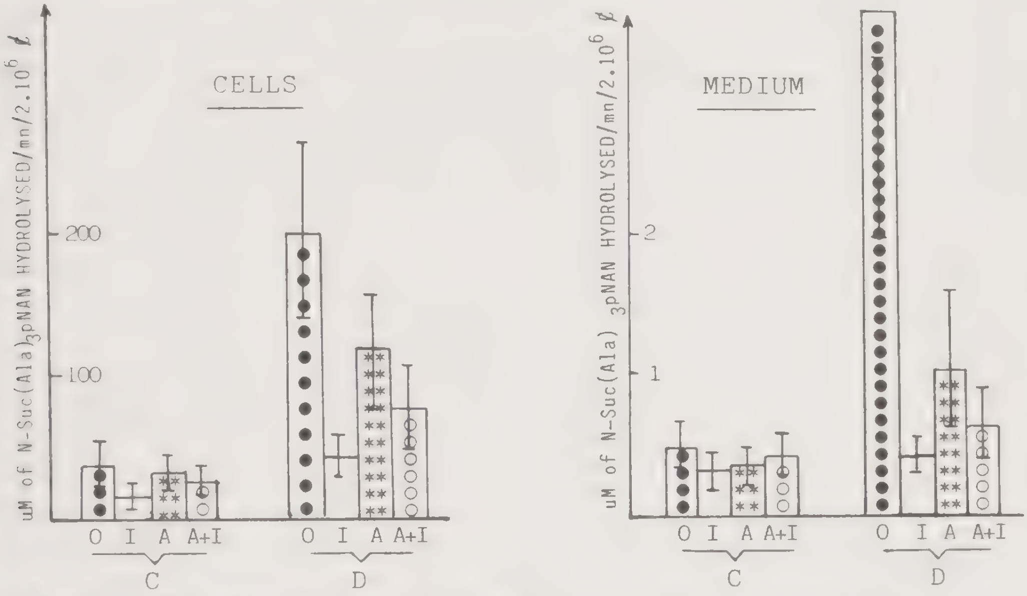


Fig.4. Biosynthesis and exportation of elastase by aortic SMC; Effects of insulin and anthocyanosides.

ANTHOCYANOSIDES AND THE BLOOD-BRAIN BARRIER

"Passive" capillary permeability is an osmotic function; it depends on the capillary basal lamina.

"Active" capillary permeability is dependent upon endothelial cells; it functions by opening and closing of intercellular junctions and by transfer of vesicles of pinocytosis.

To study these two functional aspects, the blood-brain barrier is a material of choice. In fact, certain molecules cannot be transported except by pinocytosis because a "cement" makes the intercellular junctions impermeable to these molecules.

A.M. ROBERT et al. /4/ demonstrated that a single injection of collagenase (100 µg) into rat cerebral ventricles provoked passage into the cerebral parenchyma of a tracer (trypan blue) injected into the veins of the animal one hour after the collagenase (evaluation by colorimetry).

Simultaneously, the level of hydroxyproline was increased in the cerebrospinal fluid, resulting from the degradation of collagen in the capillary basal laminae; it passed from 1.5 µg/ml (controls) to 6.7 µg after collagenase.

Treatment of animals with anthocyanosides of VM before the injection of collagenase, reduced by 25% the passage of the dye into the cerebral parenchyma and by 28% the level of hydroxyproline liberated into the CSF, when compared with untreated controls.

Reduction in liberated hydroxyproline in the CSF is proof of interaction of the VMA with the collagen of the capillary basal laminae, but reduction of the permeability of the BBB also proves that an action exists on endothelial pinocytosis.

The use of horse-radish peroxidase enables observation of the pinocytosis vesicles in the endothelial cells, and to count them and measure them. The mean diameter of the vesicles and the mean volume transported were evaluated by an image analyser. In the table below (Table 3) it can be seen that collagenase considerably increased these two permeability factors.

Table 3

Numerical density, volumetrical fraction and mean diameter of detected HRP particles in endothelium cells with NS 2000.

N_V $(1/u^3)$	130 Å	200 Å	250 Å	350 Å	500 Å	800 Å	1100 Å	1500 Å	TOTAL
Control	0	5 ± 4	8 ± 4	5 ± 3	7 ± 6	0	0	0	25 ± 17
Coll.	10 ± 3	7 ± 2	27 ± 5	31 ± 7	11 ± 2	5 ± 2	3 ± 1	1 ± 5	95 ± 22

V_V : Control 0.005 ± 0.004 – Coll. 0.05 ± 0.02
$\bar{D}$ (Å):Control 330 ± 47 – Coll. 364 ± 82

In the following graph, it can be seen that treatment with VMA (5 mg/d per 100 g of animal for ten days as intraperitoneal injections), before the collagenase or the pronase, restored endothelial pinocytosis to normal.

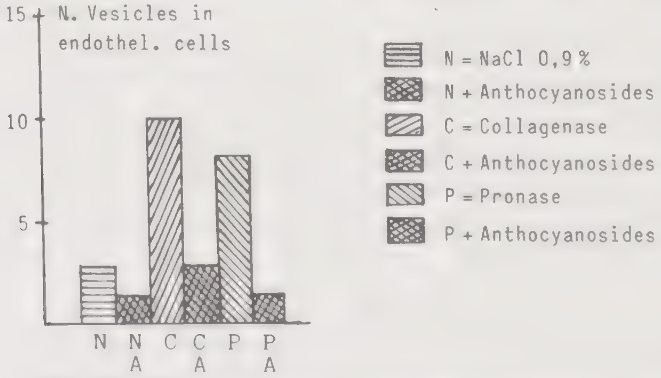


Fig. 5. The columns represent the mean number of pinocytotic vesicles in endothelial cells.

Pharmacological studies to assess the effects of anthocyanosides have demonstrated:

- A reduction in biosynthesis of polymeric collagen and of structural glycoproteins, responsible for thickening of capillaries of diabetics.

- A reduction in elastolytic activity, increased in diabetes.

- A reduction in pinocytotic function of BBB, experimentally increased by the adminstration of protease. Note that the blood-brain barrier, similar to the BBB, is also made permeable by diabetes.

These pharmacological effects could suggest the interest of using VMA for the treatment of diabetic vasculopathy, but results of a study conducted by G. LAGRUE et al., published in part /1/, enable correlation of pharmacological and clinical findings.

54 patients, who were diabetic and often hypertensive, were treated with VMA (500 to 600 mg/day for 8 to 33 months). Seven out of ten, these patients had already been treated for their diabetes or their hypertension with conventional treatments (including 6 with an oral hypoglycemic and 3 with insulin) but various microvascular lesions still persisted.

The following examinations were carried out on these patients:

- Isotopic biochemical studies on skin and gum biopsy specimens.

- Determination of thickness of capillary basal laminae in dermis.

- Conjunctival biomicroscopy examinations showing the appearance of conjunctival microvessels (dilatations, microaneurysms) and features of blood-flow (rate, fractioning, sludge).

- Ophthalmological examinations (retinoscopy and fluorography).

These latter examinations allowed classification of the patients into three groups:

- A group with specifically diabetic retinopathy (N=17);

- A group with diabetes and hypertension and signs of hypertensive retinopathy, with or without associated signs of diabetic retinopathy (N=26);

- A group of hypertensive diabetics without retinopathy (N=11).

All biochemical tests conducted (N=19) provided results showing connective tissue lesions previously reported in other studies on diabetics /2/.

All patients examined by histology (N=35) had thickening of capillary basal laminae of the dermis.

Of the 54 patients examined by conjunctival biomicroscopy, 52 had diabetic type lesions (including 33 with microaneurysm).

The results of treatment were as follows:

Isotopic biochemistry showed that treated diabetics (Table 4), compared amongst themselves and to a series of normal controls, presented:

- Almost total normalization of polymeric collagen (TCA extract).

- A reduction of 30% in structural glycoprotein (Urea extract).

- No modification in total soluble proteins (CTC extract).

These results are comparable with those obtained by cell pharmacology.

Table 4
Anthocyanosides activity on diabetes:
Isotopic biochemistry
The measurement of ^{14}C proline incorporated during a given time represents the quantity of synthetised collagen.

Extracts	Controls	Diabetics	Diabetics + Antho
CTC	73.72 ± 8.74	27.93 ± 3.28	24.87 ± 4.88
TCA	4.61 ± 0.44	16.21 ± 2.34	5.42 ± 1.00
Uree	0.88 ± 0.05	2.58 ± 0.19	1.83 ± 1.01

CTC contents total soluble proteins, included soluble collagen.
TCA contents insoluble collagen.
Uree contents structural glycoproteins.

With respect to histological examinations of cutaneous microangiopathy 27 patients accepted a second biopsy. In 16 the thickness of the basal laminae had diminished to varying degrees, while in 8 it was unchanged and in the three others it had increased.

A further study was conducted by J.D. ROMAND /5/ with a method of evaluation of microangiopathy which diminished the role of subjective factors: counting of affected capillaries and conversion into a mean percentage measured micrometrically on various diagonals of the thickness of the basal laminae, and calculation of the mean "corrected area".

Of 32 diabetics treated, the mean number of capillaries with lesions changed from 34% before to 14% after treatment. With respect to the "corrected area" of the basal laminae, this changed from an index of 14 to an index of 8 for the total examinations, the number unchanged being 9.

Treatment was by administration of 600 mg/day of anthocyanosides over 6 months.

These results correspond to reductions in biosynthesis of polymeric collagen and of structural glycoproteins observed, both in treated diabetics and in cell cultures.

Conjunctival biomicroscopy in 53 diabetics before and after treatment produced data indicating globally, normalization in 20 cases (37%), improvement in 27 (50%) and no change in 6.

Enumeration of microaneurysms, which can be conducted methodically, over a large area of the bulbar conjuctiva is definitely the sign the least open to subjective interpretation in this type of examination.

We found that in the 33 patients with microaneurysms, these had totally disappeared in 24 cases (72%), were reduced in number in 8 cases and unchanged in 1 case.

Ocular fundus examinations were carried out by Prof. COSCAS.

15 patients were re-examined after treatment by retinoscopy, combined in 11 cases with fluorescein angiography. Retinoscopy showed improvement in 10 cases and no change in 5. Fluorography showed improvement in 5 cases and no change in 6, in agreement with the corresponding results of retinoscopy.

23 of the diabetic hypertensive patients were followed up after treatment. They tended to show signs of hypertensive retinopathy but all presented other signs of diabetic microangiopathy (histology, conjunctival biomicroscopy) and biochemical modifications of diabetes.

Retinopathy was improved in 13 cases and unchanged in 10. The results in this case were not so good, but association of diabetes and hypertension is a definite handicap until blood pressure has been restored to normal.

CONCLUSION AND REMARKS

Experimental pharmacological results obtained at the cellular level by the use of anthocyanosides of VM are in agreement with clinical results and partially explain the mechanisms.

But it should be noted that the clinical results were obtained:

- 1) With the association of conventional treatments for diabetes and hypertension, previously administered to 7 of 10 cases before addition of VMA; it would be imprudent to deprive patients of the advantages they supply.

- 2) By the administration, for the first time as far as we know, of anthocyanosides for prolonged periods (8 to 33 months) and at sufficient doses (500 mg/day as two divided doses), and not at low doses (200 mg/day) as discontinous therapy over a short period.

When facing a disease such as diabetes, means for its treatment cannot be limited.

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NEW RESULTS IN THE PHARMACOLOGY OF BENZOPYRONE DERIVATIVES AND RELATED COMPOUNDS

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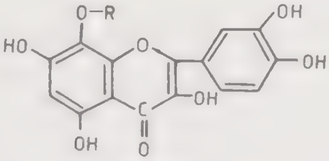
INTRODUCTION

At the most recent international bioflavonoid symposium (6th Hungarian Bioflavonoid Symposium, Munich, 1981), I gave an account of the pharmacology of benzopyrone derivatives (Gábor, 1982).

In this lecture I shall deal with the latest results of the pharmacological research on benzopyrone derivatives and related compounds. A detailed survey of the research results has recently been published (Gábor, 1985).

I. PHARMACOLOGY OF THE CENTRAL NERVOUS SYSTEM

Analgesic activity of gossypin

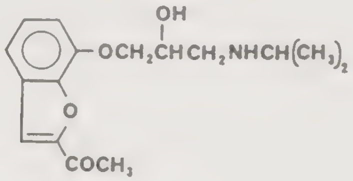


Gossypin

Viswanathan et al. (1984) studied gossypin-induced analgesia in mice, using the acetic acid-induced writhing test. Gossypin was administered in varying doses (12.5, 25, 50 and 100 mg/kg i.p.) 60 min prior to acetic acid challenge.

In all the doses tested, gossypin significantly reduced the number of writhings induced by acetic acid. The effect was dose-dependent and the maximum effects was observed at 100 mg/kg.

II. PHARMACOLOGY OF THE VEGETATIVE NERVOUS SYSTEM



BEFUNOLOL

2-Acetyl-7-/2-hydroxy-3-isopropylaminopropoxy/benzofuran (Befunolol; Kakenyaku Kako Co. Ltd., Kyoto, Japan) is a new β-adrenoceptor blocking drug. Befunolol has recently been introduced into ophthalmological use under the name Glauconex^R. It has value in therapy, as it decreases the aqueous humour production, and hence the intraocular pressure. Results of experiments in animals and isolated tissues indicate that the compound blocks β_1- and β_2-adrenoceptors approximately equally, being 2-7 times more active than propranolol (Takenaka et al., 1974).

III. PHARMACOLOGY OF THE SMOOTH MUSCLES

I earlier reported on the spasmolytic effects of flavoxate (Urispas; Smith, Kline and French Laboratories, Philadelphia), the tetrahydroisoquinolyl-alkoxyflavones and (N-/7-flavonyloxy/-acetyl)-tetrahydropapaverine (Gábor, 1982).

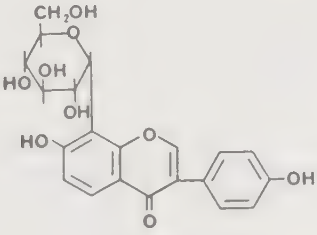
Van den Broucke et al. (1982) and van den Broucke and Lemli (1983) investigated the spasmolytic effects of the flavonoids from Thymus vulgaris L. They compared the activities of flavones from T. vulgaris and of thyme extracts with those of some standard drugs. The flavonoids from T. vulgaris were 5,4-dihydroxy-6,7,3'-trimethoxyflavone (cirsilineol), 8-methoxycirsilineol and 5,6,4'-trihydroxy-7,8,3-trimethoxyflavone (thymonin).

Flavonoids were studied in vitro for spasmolytic activity on the smooth muscles of the guinea-pig ileum and trachea and of the rat vas deferens. The flavones and thyme extracts inhibit responses to agonists which stimulate specific receptors (acetylcholine, histamine, L-noradrenaline), as well as to agents whose actions are not mediated via specific receptors ($BaCl_2$). Cumulative dose-response curves point to a non-competitive antagonism.

IV. PHARMACOLOGY OF THE CARDIOVASCULAR SYSTEM

I have previously given detailed accounts of the antianginal effects of khellin, Recordil^R (Recordati, Milan), carbochromene (Intensain; Cassella-Riedel Pharma, Frankfurt a.M.), morocromen, cinecromen, AD-6, amiodarone (Cordarone^R; Labaz, Brussels) and benziodarone (Amplivix^R; Labaz, Brussels) (Gábor, 1982,1985). I shall now deal with the latest results.

Puerarin



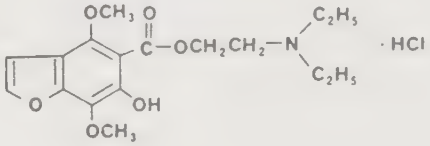
PUERARIN

Puerarin (8-D-glucopyranosyldaidzein) is an isoflavone derivative isolated from the Chinese herb Radix pueraria lobata (Willd.) Ohwi.

Radix pueraria has long been used in China as a remedy to relieve the symptoms of hypertension and angina pectoris.

However, detailed pharmacological investigations have only recently been carried out by Fan-Li-Li et al. (1982).
Intra-arterial injection of puerariae into the coronary artery of anaesthetized open chest dogs induced marked dilation of the artery without influencing the other vessels.

B-VII

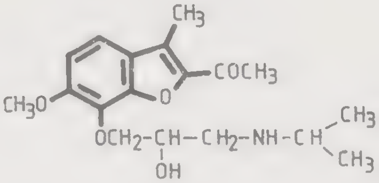


B-VII

B-VII is a new benzofuran derivative: 2-diethylaminoethyl-6-hydroxy-4,7-dimethoxy-5-benzofuranocarboxylate hydrochloride.

Woicke et al.(1981) described the effect of the compound on the circulatory system of experimental animals. The antiarrhythmic action was tested using the model of experimental arrhythmia induced in rats with $BaCl_2$; arrhythmia appeared 10-25 s after the administration of $BaCl_2$. All the animals died during the 3rd min of the arrhythmia. All rats receiving B-VII prophylactically in a dose of 5.5 mg/kg before $BaCl_2$, survived the 10-min observation period.

2-Acetyl-3-methyl-6-methoxy-7-/2'-hydroxy-3'-isopropylaminopropoxy/benzofuran



2-ACETYL-3-METHYL-6-METHOXY-7-(2'-HYDROXY-3'-ISOPROPYL-AMINOPROPOXY)-BENZOFURAN

Jedrych and Borkowska (1983) recently investigated benzofuran derivatives. The most active compound was 2-acetyl-3-methyl-6-methoxy-7-/2′-hydroxy-3′-isopropylaminopropoxy/benzofuran. The drug was active in aconitine-induced arrhythmia in rats, but its therapeutic index was much lower than that of propranolol.

Lipid-altering activity of furochromones

Gammil et al.(1983) recently described a structure-activity relationship with regard to the lipid-altering activity of furochromones (khellin analogues). The experiments were carried out with adult, male S.E.A. Japanese quail at 5-7 weeks of age. Drugs were mixed into the diet. After two weeks on the diet, the birds were bled from the right jugular vein.
Individual serum samples were analysed for HDL, VLDL and LDL cholesterol levels.

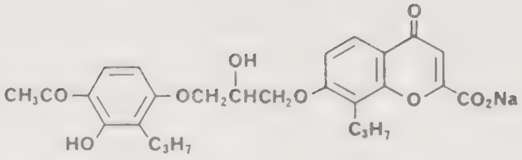
According to Gammil et al. (1983), the removal of both the C-4 and C-9 methoxy groups resulted in complete loss of lipid-altering activity. 9-Methoxyfurochromone exhibited marginal activity, while visnagin (2-methyl-4-methoxy-furochromone) was found to be equal in potency to khellin. These results indicate that, within this series, the C-4 methoxy group is required for lipid-altering activity.

V. PHARMACOLOGY OF THE RESPIRATORY SYSTEM

I earlier mentioned the effects of disodium cromoglycate and some new orally active antiallergic agents (terbucromil, proxicromil, FPL-52757, FPL-57787, AA-344, Y-12141, baicalein and EAA) (Gábor, 1982), and subsequently dealt with them in more detail (Gábor, 1985).

Here, I shall give a brief account of the antiallergic effects of FPL-55712, FPL-59257.

FPL 55,712



FPL-55,712

7(3-/4-Acetyl-3-hydroxy-2-propylphenoxy/-2-hydroxypropoxy)-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid monosodium salt (FPL 55712; Fisons Ltd.,Loughborough, England), is an antiallergic agent.

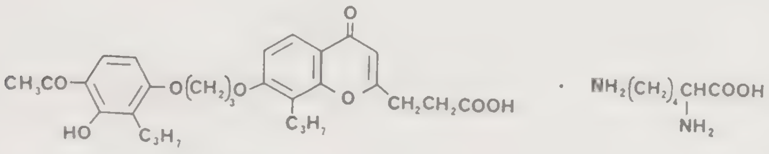
For a number of years, FPL 55712 has been used as a pharmacologic tool to ascertain the involvement of SRS-A (now known to be a mixture of leukotrienes C, D and E /LTC_4, LTD_4 and LTE_4/) in animal models of allergic asthma (Sheard, 1981).

Krell et al. (1981) produced cumulative dose-response curves to leukotriene on smooth muscle from guinea-pig trachea and bronchus antagonized by FPL 55712, but the contractile responses to leukotriene on guinea-pig parenchymal lung strips were not antagonized by FPL 55712. The compound inhibits both cAMP and cGMP phosphodiesterase (Chasin and Scott, 1978) and allergic mediator release and thromboxane synthetase activity (Welton et al., 1981). It has recently been observed to inhibit the formation of lipoxygenase products (Casey et al.,1983).

It may be mentioned here that Boyd et al. (1983) found that intracoronary LTD_4 in the dose range 0.1-3.0 µg (0.2-6.0 nmol) produced dose-dependent decreases in the coronary flow of anaesthetized pigs; pretreatment with intracoronary FPL 55712 (0.1, 0.3 and 1.0 mg) reduced the coronary constriction due to 1.0 µg LTD_4 by up to 77%.

In the experiments of O'Donnel and Welton (1984), FPL administered as aerosol was investigated for efficacy at protecting against leukotriene-induced bronchoconstrictions in guinea-pigs and for mediator release inhibitory activity in passively sensitized rats. These studies demonstrate that, when administered as aerosol, FPL 55712 is effective at protecting against leukotriene-induced bronchoconstrictions, exhibits a long duration of action, and also possesses weak anti-allergic activity.

FPL 59,257



FPL-59,257

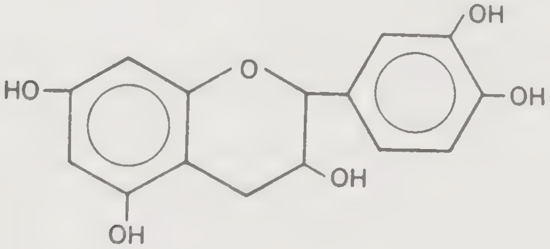
FPL 59257 (Fisons Ltd., Loughborough, England) is an anti-asthmatic agent. Its chemical structure is 3{7-(3-/4-acetyl-3-hydroxy-2-propylphenoxy/propoxy)-4-oxo-8-propyl-4*H*-1-benzopyran-2-y1} propionic acid lysine salt.

Holroyde et al. (1981, 1982) examined the possible importance of leukotrienes (LTs) as mediators of bronchoconstriction. Freshly prepared solutions of LTC and LTD (10 µg/ml) were first administered by aerosol to assess the cough response induced by these compounds, as well as the bronchoconstrictor response. Subsequently, both subjects received aerosol inhalations at intervals of disodium cromoglycate, FPL 55712 and FPL 59257 (3 mg/ml). Both FPL compounds abolished the cough response, and also inhibited to a considerable extent the bronchoconstrictor response, but FPL 59257 proved to be the more effective.

Anti-ulcer activity of benzopyrone derivatives

I earlier reported on the ulcer-inhibiting effects of gossypin and cianidanol (Gábor, 1982). I now deal with the newest results concerning the anti-ulcer activity of cianidanol, 3-O--methyl-/+/-catechin, apigenin-7,4'-di-O-methyl ether, SU-88, naringenin and hypolaetin-8-glucoside.

Cianidanol



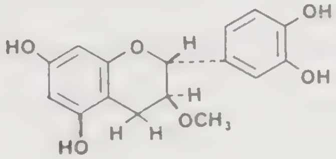
(+)-CYANIDANOL-3

Cianidanol is 2R,3S-3,5,7,3',4'-pentahydroxyflavan (/+/-catechin, /+/-cyanidanol-3; Catergen^R, Zyma-Blaes AG, Munich, FRG).

A detailed account of the anti-ulcer activity of /+/-cyanidanol-3 has been reported by Parmar and Ghosh (1981). The effect of /+/-cyanidanol-3 was studied on the gastric mucosal damage induced by aspirin, phenylbutazone, indomethacin, ibuprofen and reserpine in rats.Pretreatment with /+/-cyanidanol--3 (doses: 3 x 100, or 4 x 100 mg/kg, orally) decreased the incidence as well as the mean intensity score of the mucosal damage.

Parmar and Ghosh (1981) concluded that the gastric anti-ulcer activity of /+/-cyanidanol-3 may be due to "/i/ its antihistidine decarboxylase activity, /ii/ inhibition of the degranulation of mast cells, /iii/ direct antagonism of the effects of histamine on the capillaries of the gastric mucosa, and /iv/ a combination of more than one of these mechanisms".

3-O-Methyl-/+/-catechin



3-METHOXY-5,7,3′,4′-TETRAHYDROXY-FLAVAN

The compound 3-O-methyl-/+/-catechin (/+/ 3′,4′, 5,7-tetrahydroxy-3-methoxyflavan), an analogue of /+/-cyanidanol-3, has recently been reported by Parmar and Ghosh (1982) to be effective against gastric ulcer. The effects of this flavan derivative were studied on various types of experimentally induced gastric ulcers in rats: pylorus ligated and restraint ulcers and gastric mucosal damage induced by aspirin, phenylbutazone and reserpine.

Albinus et al. (1983) recently described histidine decarboxylase inhibition by 3-O-methyl-/+/-catechin and gastric secretion in the cat. In conscious cats provided with chronic gastric fistulas, gastric secretion was stimulated for 3 h with pentagastrin (6 µg/kg/h i.v.), insulin (0.2 IU/kg/h i.v.) or food.

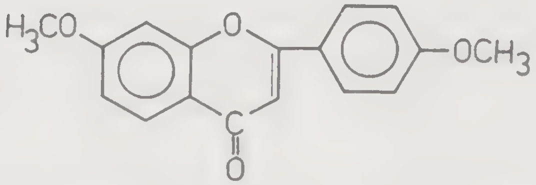
Acid output following pentagastrin stimulation 30 min after pretreatment with 200 mg/kg catechin derivative increased significantly during 1 hr of stimulation. In contrast, pretreatment with the same dose 90 min before stimulation reduced the 1-h acid output by about 40%. Lower doses were ineffective.

The response to insulin was significantly reduced by about 50% during the 1 h of secretion following direct pretreatment with 25 or 200 mg/kg. When the flavonoid compound was given 30 min before stimulation, the acid output was dose-dependently reduced. The maximum reduction in acid output by about 70% per h was achieved with 50 mg/kg.

Food-stimulated acid secretion was dose-dependently reduced when 3-O-methyl-/+/-catechin was given 90 min before stimulation. The maximum reduction in acid output was about 50%, and was achieved with 25 mg/kg. This dose caused no disturbing side-effects.

According to Albinus et al. (1983), the specificity of the catechin derivative as histidine decarboxylase inhibitor should be established in isolated intact gastric mucosal cell preparations to exclude neuronal effects; dose-dependent side-effects of 3-O-methyl-/+/-catechin might have been due to histidine decarboxylase inhibition in the brain and changes in histaminergic neurotransmission.

Apigenin-7,4′-di-O-methyl ether



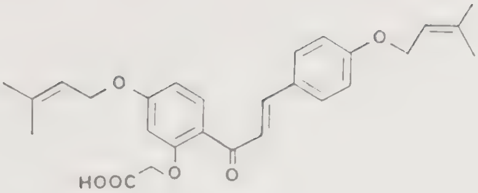
APIGENIN 7,4′-DI-O-METHYL ETHER

The effects of apigenin-7,4′-di-O-methyl ether, a flavone isolated from Andrographis paniculata (Acanthaceae), on experimentally induced ulcers has been described by Viswanathan et al. (1981).

The anti-ulcer effect was studied in Shay rats, on aspirin-induced ulcer in guinea pigs.

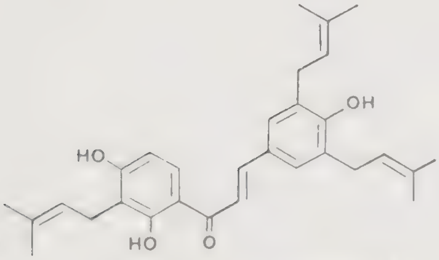
Pretreatment with the apigenin derivative (10 or 50 mg/kg) significantly reduced ulcer formation in Shay rats or in response to histamine or aspirin treatment.

SU-88

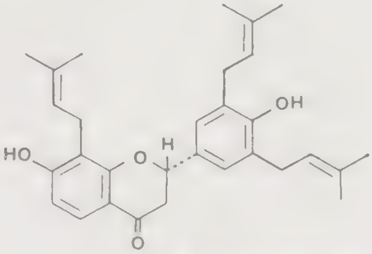


SU-88

SU-88 (Taisho Pharmaceutical Co. Ltd. Ohmiya, Japan) is 2′-carboxymethoxy-4,4′-bis/3-methyl-2-butenyloxy/chalcone, a new anti-ulcer agent. The compound is related to sophoradin isolated from Guang-don-gen, a Chinese crude drug made from the root of Sophora subprostata CHUN et T. CHEN (Leguminosae). It has been used in Chinese Medicine in the treatment of various gastrointestinal diseases. Screening of a series of fractions from S. subprostata on pylorus-ligated and stressed rats led to the identification of the fraction having the most potent anti-ulcer activity. Sophoradin and sophoranone, two isoprenylchalcones, were active components isolated from this fraction, but the former was the most potent in inhibiting free and the total output of gastric juice (Sasajima et al., 1978; Kyogoku et al., 1979).

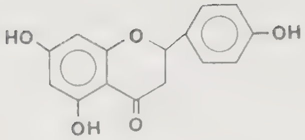


(I) Sophoradin



(II) Sophoranone

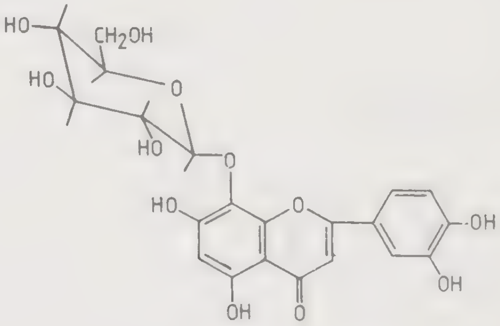
Naringenin



NARINGENIN

Parmar (1983) studied the gastric anti-ulcer activity of a specific histidine decarboxylase inhibitor, naringenin, a naturally occurring flavanone glycoside obtained from kino fruit and grapefruit, on various types of ulcers experimentally induced in rats, viz. pylorus-ligated and restraint ulcers, and on the gastric mucosal damage induced by aspirin, phenylbutazone or reserpine. Naringenin possessed significant anti-ulcer activity in all these models, manifesting a dose-dependent anti-ulcer effect on pylorus-ligated and restraint ulcers.

Hypolaetin-8-glucoside



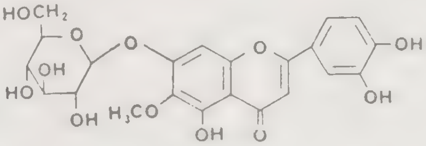
HYPOLAETIN-8-GLYCOSIDE

Villar et al. (1984) carried out investigations on the anti-ulcer property of a novel flavonoid, hypolaetin-8-glucoside, obtained from Sideritis mugronensis. Hypolaetin-8-glucoside was compared with cimetidine for its ability to prevent the formation of gastric lesions in the stomachs of starved rats subjected to 4 h cold-restraint stress. Both compounds exerted strong protective effects, which were significant. Cimetidine was considerably more potent, with a calculated ED_{50} value of 23.9 ± 11.8 mg/kg i.p., compared with 57.3 ± 11.7 mg/kg for the flavonoid.

VII. PHARMACOLOGY OF INFLAMMATION

In recent years I have dealt in detail with the anti-inflammatory action of benzo-γ-pyrone derivatives (flavonoids) on a number of occasions (Gábor 1972, 1975, 1979). Here I wish primarily to survey the most recent results relating to this theme.

Nepitrin



NEPITRIN

The anti-inflammatory action of nepitrin 5,3′,4′-trihydroxy-6-methoxyflavone glucoside, obtained from Nepeta hindostana has recently been reported by Agarwal (1982). The anti-inflammatory efficacy of nepitrin was investigated in both acute and chronic models of inflammation in rats. 20 and 40 mg/kg nepitrin inhibited the carrageenin-induced oedema by 54% and 65% ($p < 0.01$), respectively.

The anti-inflammatory effect of nepitrin on mycobacterium adjuvant-induced arthritis was studied as well. Like phenylbutazone, nepitrin in a dose of 20 mg/kg inhibited the inflammation from the 3rd day onwards. Nepitrin and phenylbutazone inhibited the inflammation to almost the same extent (75% and 77%, respectively).

Agarwal (1982) states that nepitrin may be useful as an anti-inflammatory and anti-arthritic agent.

Hypolaetin-8-glucoside

Villar et al. (1984) recently described the anti-inflammatory property of this novel plant flavonoid, obtained from *Sideritis mugronensis*, using the adjuvant-carrageenin-induced inflammation model in male Wistar rats. Test drugs were injected 1 h before and 24 h after carrageenin. The anti-inflammatory activity of hypolaetin-8-glucoside was compared with that of phenylbutazone. Both compounds suppressed oedema in the acute phase in a dose-dependent manner, but the flavonoid was more potent.

Villar et al. (1984) also obtained evidence that hypolaetin-8-glucoside not only prevents oedema, but is comparable in efficacy to phenylbutazone in inhibiting the development of granulation tissue in the carrageenin-abscess model.

As to the mechanism of action of hypolaetin-8-glucoside, in preliminary experiments using sheep seminal vesicle microsomes Alcaraz and Hoult (cit. Villar et al., 1984) have found no evidence that this flavonoid inhibits cyclooxygenase, although phenylbutazone and indomethacin actively did so (unpublished observations).

As I have earlier mentioned, the flavonoid and cimetidine both prevented the formation of cold-restraint-induced gastric lesions. According to Villar et al. (1984), these results show that hypolaetin-8-glucoside combines both anti-inflammatory and anti-ulcer properties, and suggest that it may offer useful alternatives to anti-inflammatory drugs of the aspirin type.

CONCLUSION

The most important results in flavonoid research during the past ten years may be summarized as follows:

1. The pharmacokinetics have been established for various flavonoids which can be applied as drugs or in drug combinations: O-/β-hydroxyethyl/rutosides (Venoruton^R, Paroven^R, Zyma S.A., Nyon, Switzerland), cianidanol (/+/-catechin, Catergen^R, Zyma S.A., Nyon, Switzerland), silymarin (silybin, Legalon^R, Madaus, Cologne, FRG), diosmin (Daflon^R, Laboratories Servier, Gidy, France), coumarin (Venalot^R, Schaper and Brummer, Salzgitter-Ringelheim, FRG), flavoxate (Urispas^R, Smith, Kline and French, Philadelphia, USA), etc.

2. Some enzyme-influencing effects of the flavonoids have become known, relating to lens aldose reductase, catechol-O-methyltransferase, cyclic-AMP phosphodiesterase, histidine decarboxylase, prostaglandin synthetase, lipoxygenase, etc. These results may promote the understanding of the mechanisms of action of the various pharmacological effects.

3. The discovery of some new anti-ulcer agents appears to be of significance from both pharmacological and therapeutic aspects. Mention should primarily be made of 3-O-methyl-/+/-catechin and a chalcone derivative: 2'-carboxymethoxy-4,4'-bis/3-methyl-2-butenyloxy/chalcone (SU-88, Taisho Pharmaceutical Co. Ltd. Ohmiya, Japan).

4. Special mention should be made of the development of new antiallergic agents that are also effective orally.

5. Recent research has led to the discovery of new flavonoids of natural origin with anti-inflammatory effects: nepitrin, hypolaetin-8-glucoside.

All these results strongly suggest that in the future further pharmacologically effective naturally-occuring flavonoid derivatives will be discovered; that these and compounds with related structures synthetized chemically, may find therapeutic applications.

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ISOFLAVONES AND ARTERIOSCLEROSIS

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Among cardiovascular diseases arteriosclerosis occupies one of the leading positions causing a decrease in ability to work, disablement and high mortality. Hyperlipidemia is one of the essential factors responsible for the development of arteriosclerosis.

Hypolipidemic agents used in medicine at present have limited effects (Linetol, Polysponin, Cetamiphen) or side effects (D-thyroxine, β-sitosterol, Miscleron, etc.). Thus most of the available antiarteriosclerotic preparations do not meet the high requirements of practical medicine.

Naturally occurring flavonoids, especially isoflavone derivatives appeared to be rather effective for the treatment of arteriosclerosis.

Studies of the flavonoid contents of plant raw materials have shown that some Fabaceae plants, namely the species Trifolium, Astragalus, Onobrichis, Cicer and Clycine, widely employed as fodder crops, are the richest source of flavonoids (isoflavones).

From the plants investigated, 29 compounds of flavonoid nature - glycoside derivatives of kaempferol, quercetin, formononetin, biochanin A, daidzein, genistein,luteolin - have been isolated. The structures of the compounds isolated were identified by classic chemical and modern physicochemical methods. The plant material studied contained 1.5%-5% of the flavonoid compounds (1-3).

Individual flavonoid compounds isolated and complex polyphenol preparations were investigated for antiarteriosclerotic activity. To clear up the degree of hypolipidemic activity of these compounds we employed a screening model of experimental hyperlipidemia induced in white rats by injecting Triton WR-1339. The total rat serum cholesterol and trigyceride concentrations indicated the hypolipidemic effect of the substances studied.

Rats were injected 150-200 mg/kg body weight of the substances investigated intraperitoneally. The antiarteriosclerotic effect of these substances was compared with that of Miscleron, Polysponin and Cetamiphen. The experimental data obtained were treated statistically ($P<0.01-0.001$). The complex flavonoid preparations containing isoflavones proved to produce a marked hypolipidemic effect. Flavonoid preparations from both Trifolium protense and Cicer, as well as an individual isoflavone - biochanin-A glycoside - revealed a high hypocholesterinemic activity, the cholesterol decrease being 54.0, 57.3 and 53.3%, respectively. The triglyceride content decreased by 54.1, 73.3 and 51.3%, respectively. The decrease percentages produced by the substances studied were much higher than those obtainable with the officinal preparations Miscleron, Polysponin and Cetamiphen. Our flavonoid preparation "Trisoflan" is under clinical trial at present (4-7)

In view of the limited occurrence of natural isoflavones and their laborious isolation in sufficient quantities for practical purposes, we developed some methods for the synthesis of different 3-phenylchromone (isoflavone) derivatives.

o-Hydroxydeoxybenzoins, were employed as starting materials; these were prepared:

(a) by condensation of arylacetic acid anhydrides with the appropriate phenols. Anhydrous $AlCl_3$ catalyzed the reaction;

(b) by the Houben-Hoesch method: condensation of arylacetic acid nitriles and phenols by passing dry HCl through the reaction mixture, followed by hydrolysis;

(c) by the Nencki reaction (nuclear acylation of phenols with acids in the presence of anhydrous $ZnCl_2$).

To extend the set of isoflavones, some of them were synthesized by oxidative rearrangement of o-hydroxychalcones prepared by the alkaline condensation of *o*-hydroxyacetophenones and aromatic aldehydes.

We used a new method of isoflavone synthesis based on the interaction of *o*-hydroxydeoxybenzoins and an excess of ethyl orthoformate, an equimolar amount of 70% $HClO_4$ being added to the reaction mixture. The resulting perchlorates on treatment with water readily gave the isoflavones. Optimal synthesis conditions were found. They allowed to increase the isoflavone yields from 33% to 70-75% calculated for the initial *o*-hydroxydeoxybenzoin (8, 9).

There is no information on the antiarteriosclerotic effect of isoflavone hetero-analogues in the literature. Hence, to reveal perspective compounds in this series having marked antiarteriosclerotic activity, we carried out several syntheses where the initial substances were α-heteroaryl-polyhydroxyacetophenones containing various substitutents in the nucleus.

The syntheses were performed according to the Hoesch reaction. From the synthesized α-heteroarylacetophenones isoflavone analogues with different C_3-heteroaryl- and C_2-substituents were prepared. Acid and salt derivatives were prepared from these compounds (10). To find perspective hypolipidemic isoflavone derivatives, we studied the correlation between the structure and activity of natural isoflavones and of the synthetic compounds prepared. That was done with the help of the regressive (statistic) method of Free and Wilson.

Data analysis of the contribution of the nucleus and the substituents to the hypolipidemic activity of 3-phenylchromone derivatives showed the relationship between their molecular structure and biological activity. Important units best contributing to the biological effect proved to be the oxygen atom of the carbonyl group and the pyrone nucleus carrying a partial π-charge and oxygen at C_7. The presence at C_7 in the isoflavone ring system of OH, OCH_3 or OCH_2R substituents exerting a positive mesomeric effect on the conjugated carbonyl group of the heterocycle, decreased the activity to some extent. The presence of an ester group ($-OCOCH_3$) or phenoxy group (C_6H_5O-) at C_7 decreases the electron density of the carbonyl oxygen, which results in enhanced biological activity. The presence of electron-attracting substituents (OH, OCH_3) at the 5, 8 or 4′-positions considerably increase the desired effect.

These structures are certain to open up wide opportunities for further studies in the field of the synthesis of new antiarteriosclerotic agents in the series of 3-aryl-/hetero-aryl/chromone derivatives.

On the basis of these studies it is concluded that naturally occurring and synthetic isoflavone derivatives and their hetero-analogues are rather perspective new agents for the treatment and prevention of arteriosclerosis.

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THE EFFECT OF FLAVONOIDS ON THE NATURAL KILLER ACTIVITY OF HUMAN LYMPHOCYTES

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INTRODUCTION

Flavonoids have various biological effects. They are very common in nature, mainly in food of plant origin of humans and animals. Therefore their influence on the immunocompetent cells of the hosts seems to be interesting. It was shown by Busse et al. /1/ that flavonoids inhibited the neutrophil chemiluminescence response to opsonized zymosan. Quercetin, but not morin and rutin inhibited the concanavalin A-induced enhancement of oxygen consumption, without impairing leucocyte viability and concanavalin A binding /2/. Quercetin also inhibited histamine release from mucosal mast cells /3/. In this paper we present experiments in which the effect of quercetin, hesperidin and rutin on the in vitro natural killer (NK) cell activity was tested. Quercetin, but not rutin and hesperidin inhibited the human NK cytotoxicity, and antagonistic effects of quercetin and interferon on modulating NK cell activity was found.

MATERIALS AND METHODS

Chemicals. Quercetin (Merck), hesperidin and rutin (Serva) were commercial products. A stock solution of 50 mM of each flavonoid was prepared in dimethyl sulphoxide (DMSO, purchased from Sigma).

Lymphocytes. Human peripheral blood lymphocytes (PBL) of normal donors were prepared on Ficoll-Uromiro gradient centrifugation according to the technique of Böyum /4/. The lymphocytes were resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS) at a final concentration of $5x10^6$ cells/ml. Lymphocytes were incubated in the presence of 0.25 mM quercetin, 0.25 mM rutin or 0.5 mM hesperidin for 1 h, then washed before use in the NK assay.
Interferon alpha was a product of EGIS (Budapest). Its specific activity was $1x10^6$ International Reference units per mg protein.
NK activity assay. K-562 cell line derived from a human myeloid tumour was used as target in the NK activity assay. Target cells were labelled with ^{51}Cr (Amersham, 100-150 µCi, 30 min, 37 °C) and $1x10^4$ cells, suspended in 100 µl, were transferred to U plates for cytotoxic assay. For culture, the cell mixtures were prepared in 200 µl RPMI 1640 with 10% FCS at 50:1, 25:1, 12:1, 6:1 lymphocyte to target cell ratios.

Reactions were read after a 4-h incubation (at 37 °C in a 5% CO_2 atmosphere) by determination of the supernatant activity, using the following formula for calculation of ^{51}Cr release:

$$\text{Cytotoxicity } \% = \frac{\text{c.p.m. in test - c.p.m. in control}}{\text{total incorporated acitivity}} \times 100$$

Each lymphocyte-target cell combination was set up in three parallel cultures.

RESULTS

Toxicity of quercetin on the effector and on target cells. PBL showed 98% viability by Trypan Blue exclusion test after incubation in the presence of 0.25 mM quercetin during a 18-h period. Quercetin at a concentration of 0.5 mM reduced the viability to 70% after a 4-h incubation period, and to 45-50% after 18 h. Treatment of the target cells with quercetin (0.25 mM) followed by washes did not alter subsequent NK activity with PBL.

The effect of quercetin on binding of effector and target cells. Experiments were performed to determine whether PBL binding to the target K 562 was inhibited by quercetin. Binding frequencies were identical when performed with PBL pretreated with 0.25 mM quercetin, or in medium alone for 1 h, when it was checked by phase contrast microscope.

The effect of quercetin, hesperidin and rutin on the activity of human NK cells. PBL and K-562 targets were mixed in 50:1 ratio and the kinetics of ^{51}Cr release were determined. At different times after initiation of the cytotoxicity assay quercetin at a concentration of 0.25 mM was added; the incubation was terminated after 4 h, then the ^{51}Cr release was determined. The results are summarized in Table 1.

Table 1

The effect of quercetin on NK cytotoxicity

Time of addition of quercetin after initiation of cytotoxic reaction (min)	Cytotoxicity (%) before addition of quercetin	Cytotoxicity (%) at 4 h
0	2.1	1.3
30	14.6	13.4
60	20.8	17.5
90	32.2	26.3
-	-	55.0

It can be seen that quercetin given at different times during the cytotoxicity assay exerts a blocking effect on the further increase of cytotoxicity.

Neither hesperidin, nor rutin had any effect on the NK activity (data not shown).

The effect of quercetin on the NK modulating activity of interferon alpha was also determined.

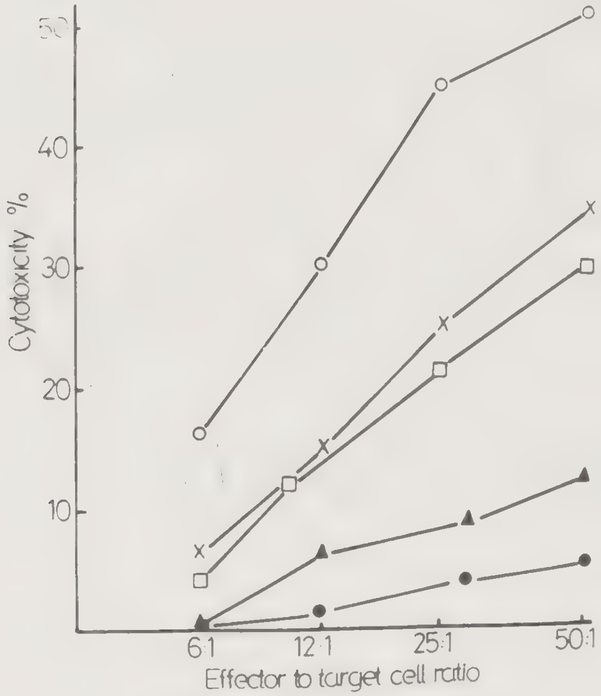

Fig.1. Effect of quercetin and interferon on NK activity x—x control lymphocytes, ●—● quercetin (0.25 mM) □—□ DMSO, o—o interferon, ▲—▲ interferon and quercetin

It can be seen (Fig. 1) that interferon (3000 IU) increased the cytotoxicity. On the contrary, quercetin at a concentration of 0.25 mM markedly decreased the NK activity. IFN and quercetin given together resulted in a similar decrease of cytotoxicity to that observed with quercetin alone.

Recovery of NK cell activity. Fig. 2 shows that NK cell activity can recover following treatment with quercetin at a concentration of 0.25 mM for 1-h.

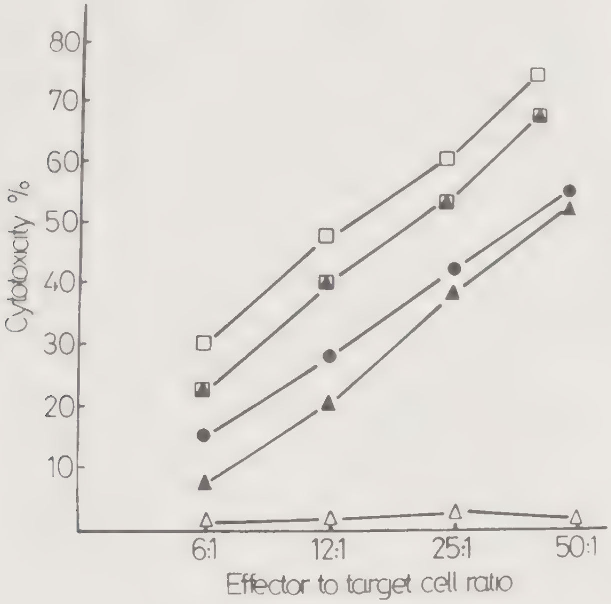

Fig.2. Recovery of cytotoxicity of quercetin-treated lymphocytes
▲—▲ quercetin treatment for 1 h, followed by washing, then further incubation in medium for 18 h
▣—▣ as above, but in the presence of interferon
●—● control lymphocytes
□—□ lymphocytes treated with interferon for 18 h
△—△ quercetin treatment for 1 h then washing and cytotoxicity determination

It is obvious that a 1-h treatment of the effector cells with quercetin abolished their NK activity. When the effector cells were washed after quercetin treatment and incubated further for 18-h, recovery of some activity can be seen. IFN (5000 IU) enhanced the NK activity either of quercetin-treated or untreated lymphocytes.

DISCUSSION

Natural killer (NK) cells are a subpopulation of lymphocytes that are defined functionally as cells that can spontaneously lyse a variety of normal and malignant cells /5/. There has been a great deal of interest in NK cells because of their possible role in mammalian host defense against spontaneous neoplastic transformations and viral infections.

The present study has shown that the in vitro natural cytotoxicity of human blood lymphocytes are inhibited by quercetin. The inhibition of cytotoxicity is due to a specific effect on the effector lymphocytes and not to an effect on the target cells. The reduction of killing is, probably, not due to the impaired target binding capacity of the effectors, but target lysis may be impaired.

There is, as yet, no biochemical evidence that defines with certainty a mechanism of action of quercetin on NK cells; superoxide radicals are possible targets for quercetin action in these cells. It has been reported /6/ that quercetin interacts with superoxide radicals and the scavenging activity may explain its action on NK cells since the generation of superoxide radicals has been implied in the NK activation mechanism /7/.

The present study investigated the interaction between quercetin and interferon in cytotoxicity. It has been found that interferon augmented NK cell activity, as shown previously /8/. When interferon and quercetin were added concurrently, the inhibition of natural cytotoxicity was as pronounced as in quercetin-treated cells. Addition of interferon to quercetin-pretreated and washed cells resulted in a better recovery of natural cytotoxicity. These findings suggest that quercetin and interferon interact at the molecular level, possibly through hydrophobic linkages.

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COMBINED ANTIVIRAL EFFECT OF QUERCETIN AND INTERFERON ON THE MULTIPLICATION OF HERPES SIMPLEX VIRUS IN CELL CULTURES

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INTRODUCTION

Previously we have reported that quercetin and other naturally occurring flavonoids have antiviral effect on herpesviruses in cell cultures /1,2/. It has also been shown that quercetin raises the cyclic AMP (cAMP) level in Ehrlich ascites tumour cells /3/. Other workers observed that cAMP enhancer compounds, such as dibutyryl cAMP, theophylline, inhibited the multiplication of herpes simplex viruses /4,5/. It was also reported that mouse interferon (IFN) induced an increase in cAMP level in IFN sensitive mouse cells and the elevation preceded the induction of antiviral activity /6/.

These data prompted us to study the antiviral effect of quercetin and human leukocyte IFN (HuIFN-α) or chicken leukocyte IFN on the yield of herpes simplex virus type 1 (HSV-1) in cell cultures.

MATERIALS AND METHODS

Viruses and cell cultures. A strain of HSV-1 was used in the experiments. HSV-1 was grown in HEp-2 cells and infectivity was measured in the same cells by the dilution method. The infective titre was expressed as $TCID_{50}$. The antiviral activity of the agents was investigated in WISH cells and in secondary chick fibroblast (CEF) cells. These cells were propagated in Eagle's basal medium as modified by Macpherson and Stoker /7/ supplemented with 5% calf serum and 10% tryptose phosphate broth.

Antiviral agents. Quercetin was a commercial product (Merck); stock solution was prepared in dimethyl sulphoxide and further diluted with culture medium. HuIFN-α was prepared in our laboratory using Sendai virus as inducer. Chicken leukocyte IFN was also produced in this laboratory and was induced by human adenovirus type 12. The IFNs used in the experiments were crude preparations.

Assay of antiviral activity. Monolayers of the WISH cells and CEF cells were grown in six well plastic tissue culture plates (Linbro, Greiner). The antiviral activity of the agents was investigated by the yield reduction test. The combined effect of HuIFN-α and quercetin on the multiplication of HSV-1 was investigated in WISH cells. After incubation of the cultures with IFN samples for 24 hours, the samples were removed and the cells infected with HSV-1 at a multiplicity of 0.1 $TCID_{50}$/cell. After adsorption for 1 hour at 37 °C, the inoculum was removed and the cultures were washed twice with Hanks' solution and supplied with medium containing quercetin. The controls received only media without quercetin. After incubation for 24 hours, the cultures were frozen and thawed, then the virus yield was determined in HEp-2 cells. The combined antiviral activity of chicken leukocyte IFN and quercetin was tested in secondary CEF cells and the virus yield was also titrated in these cells after the same procedure as described above. The virus titres were expressed as $\log_{10}$ $TCID_{50}$. A mathematical technique described by Allen et al. was used /8/ to evaluate the combined antiviral effect by means of the following procedure. The minimal inhibitory concentration (MIC) of the agents alone and in combination had to be determined. The MIC value was regarded as the concentration which caused 1 log inhibition of the virus yield and, when necessary, it was calculated by a linear regression method. The fractional inhibitory concentration (FIC) may be given by the following formula: MIC of drug in combination/MIC of drug alone. In the case of two drugs (A and B) the sum of the FIC values give the FIC index.

$$\text{FIC index} = \frac{\text{MIC of drug A in comb.}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in comb.}}{\text{MIC of drug B alone}}$$

The FIC index is useful for the interpretation of drug interactions. If the FIC index is <1, the interaction is synergic; if this index is ≈ 1, the effect is additive; if the FIC index is 1.1-1.9, the effect is indifferent or partially antagonistic, and if the FIC value is >2, the interaction is antagonistic.

RESULTS

Quercetin combined with HuIFN-α increased the antiviral effect (Fig.1), depending on the concentration.

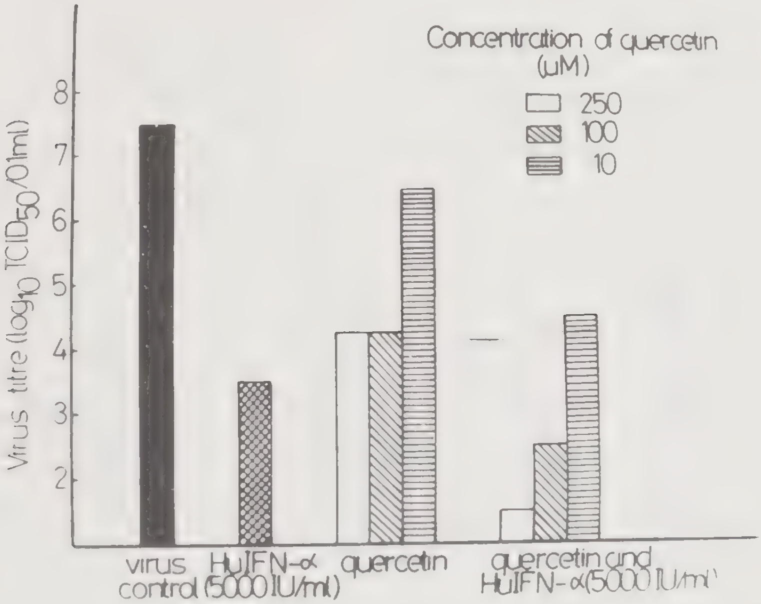

Fig.1. Combined antiviral effect of HuIFN-α and quercetin on the multiplication of HSV-1

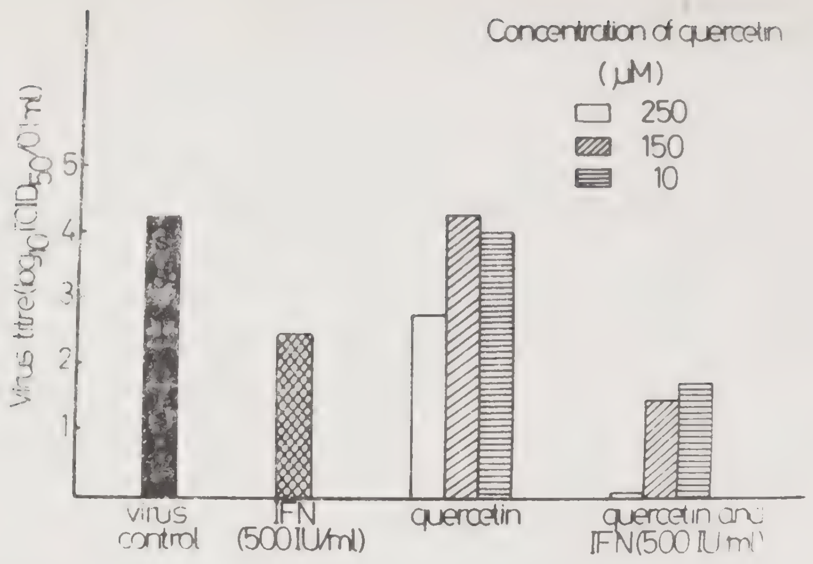

Fig.2. Combined antiviral effect of chicken leukocyte IFN and quercetin at different concentrations on the multiplication of HSV-1

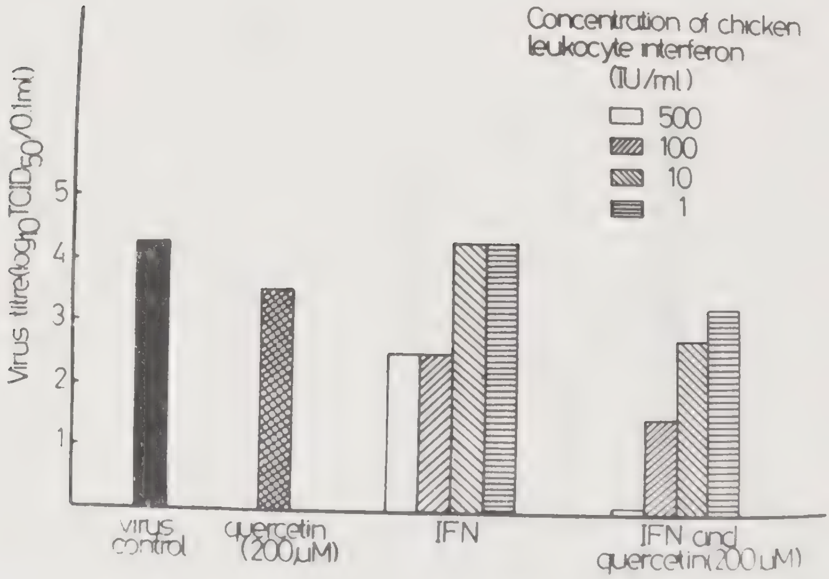

Fig.3. Combined antiviral effect of quercetin and chicken leukocyte IFN at different concentrations on the multiplication of HSV-1

The combined antiviral effect of chicken leukocyte IFN and quercetin was also investigated. The results showed that the antiviral effect of quercetin in combination is more explicit even at lower concentrations (Fig.2).
The antiviral effect of chicken leukocyte IFN at different concentrations was enhanced by 200 µM quercetin (Fig.3).
In the case of quercetin and chicken leukocyte IFN, the interaction was the most effective when 160 µM quercetin and 10 IU/ml IFN were combined; as the FIC index was 0.96≈1, an additive effect has been demonstrated.

DISCUSSION

In our earlier experiments the antiviral effect of quercetin in vitro and in vivo has been demonstrated /1,2, 9/. The mechanism of the antiviral action of quercetin is not yet known. Recently, it has been described that quercetin raises the cAMP level in Ehrlich ascites tumour cells /3/ and that flavonoids, among others quercetin, inhibit the cAMP phosphodiesterase which is responsible for the breakdown of cAMP /10,11/. We also demonstrated that quercetin and quercitrin enhanced the intracellular cAMP level in cell cultures, and a relationship between their antiviral and cAMP enhancing effect has been suggested /12/. Interferon also enhances the intracellular level of cAMP /6/, therefore a synergic effect in antiviral activity can be expected from the combination of quercetin with IFN. However, we observed an additive antiviral effect in the case of combined application of quercetin and chicken leukocyte IFN. Stanwick and Nahmias also demonstrated that the combination of IFN and cAMP enhancers had an additive effect in reducing the yields of HSV /13/. This observation supports our idea that the antiviral effect of flavonoids is based on the cAMP enhancing activity, because drugs having the same mechanism of action can have synergic or additive effect when applied in combinations.

Summarizing, it was observed that when quercetin was combined with HuIFN-α and chicken leukocyte IFN the antiviral effect against HSV-1 was enhanced. The combination of quercetin and chicken leukocyte IFN resulted in an additive antiviral effect.

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ANTIVIRAL, IMMUNOLOGIC AND TOXICOLOGIC STUDIES WITH QUERCETIN IN MICE

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Bioflavonoids, flavonoid compounds and derivatives of flavonoids have proved to be antivirally effective under in vitro (1-19) and in vivo conditions (16, 20-22). Flavonoids are ingredients of commercial pharmaceuticals recommended against common cold, and structural analogues of flavonoids have shown a favourable effect on the course of acute viral hepatitis in man (22-25). The mechanisms of antiviral activity of these agents are poorly understood. Most of what is known about the antiviral properties of flavonoids is based on experiments performed in vitro, but whether this knowledge may be applied to the intact host, is uncertain. We were therefore interested to investigate an antivirally effective flavonoid in animal model. Here we present a survey of the main results of our hitherto studies which were particularly concerned with quercetin.

ANTIVIRAL ACTIVITY OF FLAVONOIDS IN MICE

Four flavonols were examined for their antiviral activity in mice infected with Mengo virus causing severe systemic disease with fatal outcome. Repeated oral administrations of quercetin or morin protected mice significantly from lethal infection, whereas rutin and quercitrin were ineffective. When administered subcutaneously, all the four flavonols failed to prevent mortality (Table 1) (26).

Table 1

Effect of peroral (p.o.) or subcutaneous (s.c.) treatments [a] with certain flavonols on lethal Mengo virus infection in ABD2F$_1$ mice

Drug	Daily dose of drug [mg/kg]	Rate of protection and significance [b] [%]			
		p.o.		s.c.	
Quercetin	20			10.0	-
	30	22.2	-		
	40	50.0	+	0.0	-
Morin	9			0.0	-
	30	30.0	+		
	40	30.0	+	0.0	-
Rutin	18			0.0	-
	30	22.2	-		
	40			20.0	-
	55	20.0	-		
Quercitrin	14			0.0	-
	30	20.0	-		
	40	22.2	-	0.0	-

[a] Twice daily for 5 days, beginning immediately before intraperitoneal (i.p.) virus inoculation (10 LD_{50})

[b] At the 95 % level

The antiviral effectiveness of quercetin was further examined against various viral infections. As shown in Table 2 the drug was not only effective against Mengo virus infection, but also caused significant reduction of deaths in EMC-, Col.SK- and MM virus infected mice (27). In contrast, Sindbis- and herpes virus-infected mice were not protected by treatment with quercetin, suggesting a selective antiviral activity towards cardiovirus infections. Antiviral effect was observed when Mengo virus was injected intraperitoneally (i.p.) or intranasally (i.nas.), but failed to appear after intracerebral (i.c.) virus inoculation, implying that the drug did not act within the brain.

Table 2

Effect of p.o. quercetin treatment a) on different virus infections in ABD2F_1 mice

Virus b)		Survivors/total		Rate of protection and significance [%] c)	
Strain	Route of inoculation	Treated	Control		
EMC virus	i.p.	10/20	0/20	50	+
Col.SK virus	i.p.	7/20	1/20	32	+
MM virus	i.p.	8/20	1/20	37	+
Mengo_M virus	i.p.	12/20	1/20	58	+
	i.nas.	10/20	0/20	50	+
	i.c.	0/20	0/20	0	-
HSV-1	i.p.	5/20	6/20	0	-
Sindbis virus	i.p.	1/10	0/10	0	-

a) 20 mg/kg body mass at hours -1, +8, +24, +32, +48 and +56, relative to infection

b) 10 LD_{50}, calculated from the specific titres after i.p., i.nas. or i.c. titrations

c) At the 95 % level

Quercetin was used successfully to treat Mengo virus infections in several genetically different strains of mice. Infected animals were protected in a dose-dependent manner (Fig. 1). Maximum number of survivors, 50 - 60 % of infected mice, appeared at doses of 20 mg/kg body mass per administration (26).

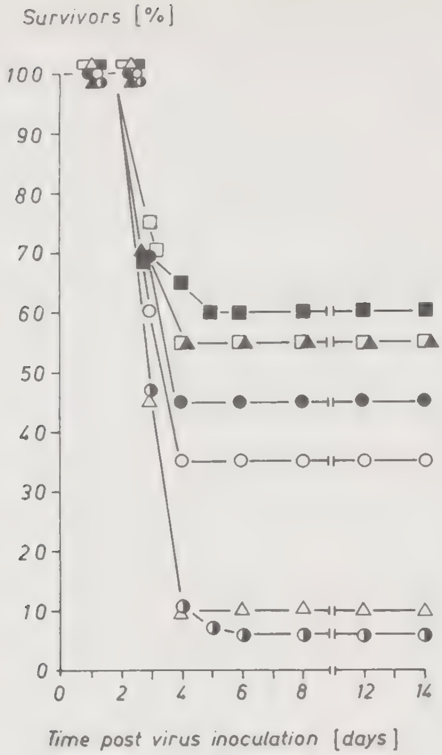

Fig. 1. Dose-response relation of quercetin in Mengo virus-infected (10 LD_{50}, i.p.) $ABD2F_1$ mice. Groups of 20 animals were infected and treated (p.o.) with placebo (◑), 5 (Δ), 10 (o) 15 (●); 20 (□); 25 (■) or 30 (▲) mg/kg body mass of the drug. Treatment was started immediately before virus inoculation and continued twice daily every 12 hours for 4 days.

To gain information on the drug-sensitive period during the course of infection, the influence of both number and initiation of treatments was studied (Table 3). When the daily dose of quercetin was given as two separate injections, significant protection was obtained. When given in a single dose the drug failed to be effective, implying a rapid degradation and/or elimination. Maximum protection appeared when the drug was administered for a period of not less than 3 days, provided that treatment was begun prior to infection. Delayed initiation of treatment was ineffective. Thus, it became clear that quercetin acts at the beginning and in the early stages of the pathogenesis of Mengo virus infection (26).

Table 3

Effect of number and time of p.o. treatments with 20 mg/kg body mass per injection of quercetin on Mengo virus infection [a] in ABD2F_1 mice

Time of treatment relative to virus inoculation [hours]	Treatment per day	Route of inoculation of virus	Rate of protection and significance [b] [%]
0, 7, 24, 32, 48, 56, 72, 80, 96, 104	2x	i.p.	50.0 +
0[c], 24[c], 48[c], 72[c], 96[c]	1x	i.p.	0.0 -
0, 7, 24, 32, 48, 56, 72, 80	2x	i.p.	47.4 +
0, 7, 24, 32, 48, 56	2x	i.p.	33.3 +
0, 7	2x	i.p.	5.3 -
0, 7, 24, 32, 48, 56, 72, 80, 96, 104	2x	i.nas.	47.4 +
0, 7, 24, 32, 48, 56, 72, 80	2x	i.nas.	45.0 +
-12[c], 0, 7, 24, 32, 48, 56, 72, 80	2x	i.p.	55.0 +
-12[c], 0, 7, 24, 32, 48, 56	2x	i.p.	57.9 +
-12[c], 0, 7	2x	i.p.	31.6 +
-12[c]	2x	i.p.	15.0 -
7, 24, 32, 48, 56, 72, 80	2x	i.p.	5.0 -

[a] 10 LD_{50} specific for either i.p. or i.nas. inoculation

[b] At the 95 % level

[c] Dose of drug was 40 mg/kg body mass

Therefore, the effect of quercetin on the spread of the virus through the body was examined during the early phase of the infection (Fig. 2).

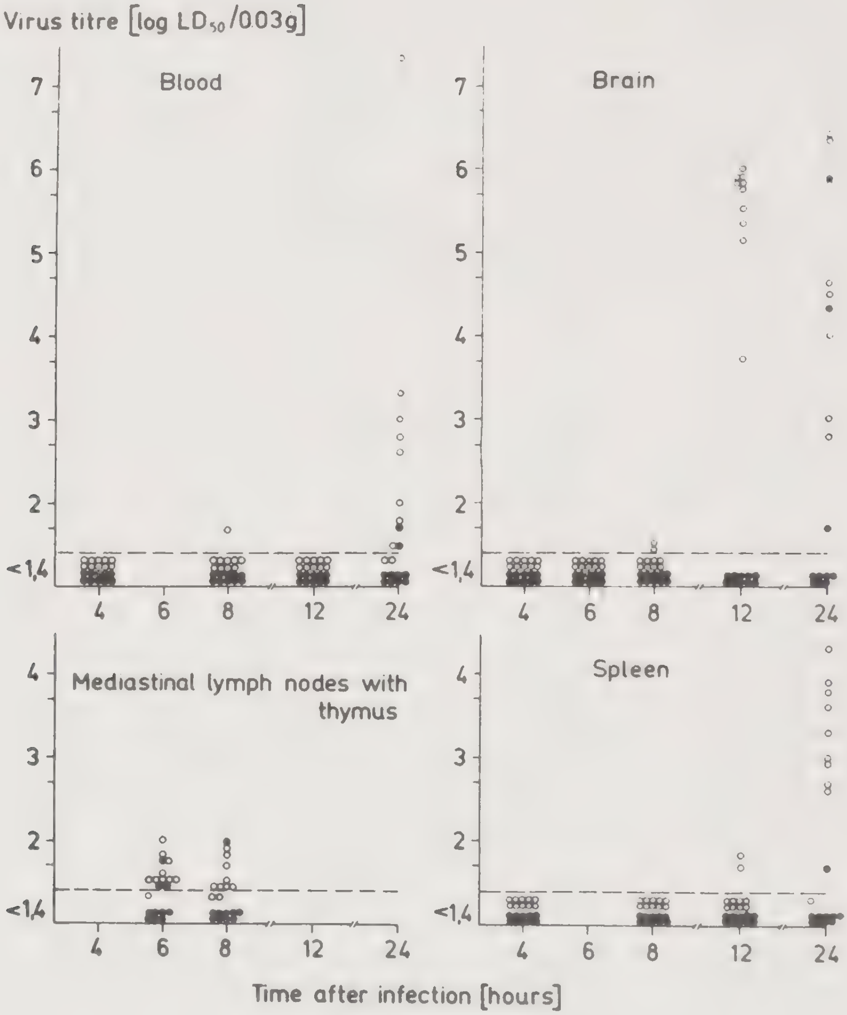

Fig. 2. Effect of orally administered quercetin on spread of Mengo virus (10 LD_{50}, i.p.) to target organs of individual mice. Placebo-treated and infected (o); quercetin-treated and infected (•)

At different times after the infection, virus content in various target organs of individual mice was determined in both placebo- and drug-treated mice. In placebo-treated animals virus was first detected in the local lymph nodes and the thymus, and thereafter in the blood, brain and spleen.

After 24 hours, the tissues of almost all mice contained virus in different amounts, but no virus could be detected in the organs of most of the drug-treated animals. These findings imply that early during the infection quercetin-treatment prevented, in most mice, spread of virus to the local lymph nodes and through the organism (27).

EFFECT OF QUERCETIN ON HOST DEFENCE

As quercetin exerted antiviral effectiveness only when present during the early phase of the infection, its effect on early host defence was investigated.

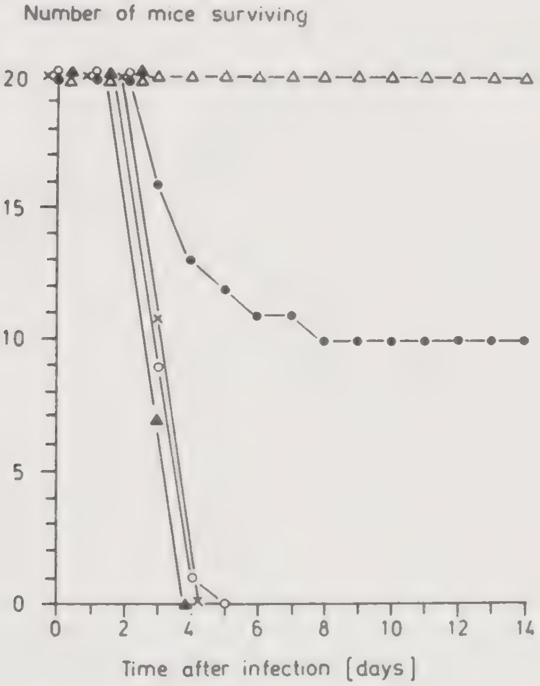

Fig. 3. Effect of silica (40 mg/kg body mass, i.p.) given 2 hours prior to Mengo virus (10 LD_{50}, i.p.), on the protective effect of quercetin in $ABD2F_1$ mice. Placebo-treated and infected (o); silica-treated and infected (x); quercetin-treated and infected (●); silica- and quercetin-treated and infected (△); silica-treated only (▲).

During 48 hours after drug-treatment no interferon was detectable in the sera of mice, indicating that the protective activity of quercetin is not due to systemic interferon induction (27).

The role of macrophages in the antiviral protection of quercetin was studied in macrophage-depleted mice (Fig. 3). Silica, a macrophage toxic agent, did not influence the course of Mengo virus infection but abolished the protective effect of quercetin completely. Thus, it appears that macrophages are essential for quercetin to induce early host resistance of mice towards cardiovirus infections (27).

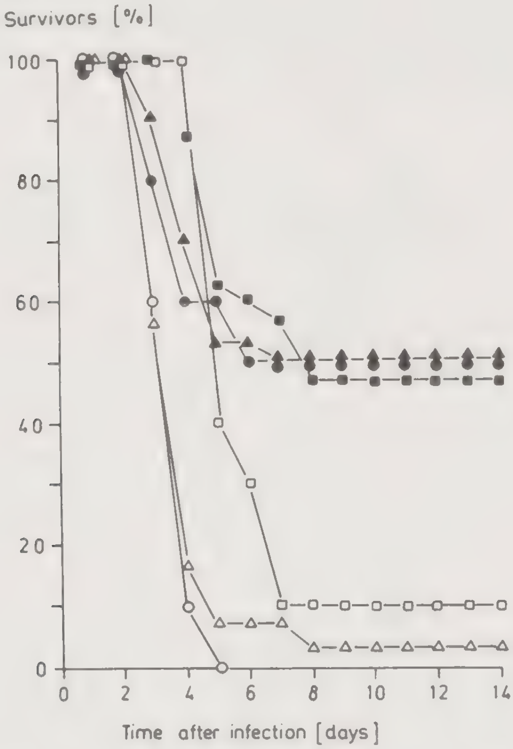

Fig. 4. Effect of quercetin (40 mg/kg body mass, p.o.) on normal and immunodeficient male C57BL/6 Jena mice infected with Mengo virus (10 LD$_{50}$, i.p.). Normal- (▲△); splenectomized- (● o); nu/nu mice (■□). Open symbols: placebo-treated; solid symbols: quercetin-treated.

Moreover, the role of the lymphatic system in the protective action of quercetin was studied (Fig. 4). The effect of drug treatment was similar in immunocompetent, splenectomized and congenitally athymic nude mice, suggesting that B- and T-lymphocytes are not involved in the antiviral effect of quercetin (28).

Interestingly, quercetin enhanced the graft-versus-host reaction, but failed to affect the humoral antibody response of mice to sheep red blood cells (28).

TOXICITY OF QUERCETIN

The general toxicity of quercetin has been found to be very low in adult male SPF mice ($ABD2F_1$, DBA/2 Jena, C57BL/6 Jena). Single oral doses up to 10 g/kg body mass were well tolerated. LD_{50} values could not be estimated with this experimental protocol; weight gain was similar as in the controls, and no organ lesions were seen by histologic examination of the animals killed 3 weeks after treatment. Likewise, antivirally effective doses of 2.5 to 50.0 mg/kg, given twice daily for four consecutive days, failed to induce any obvious toxic side effects in $ABD2F_1$ mice.

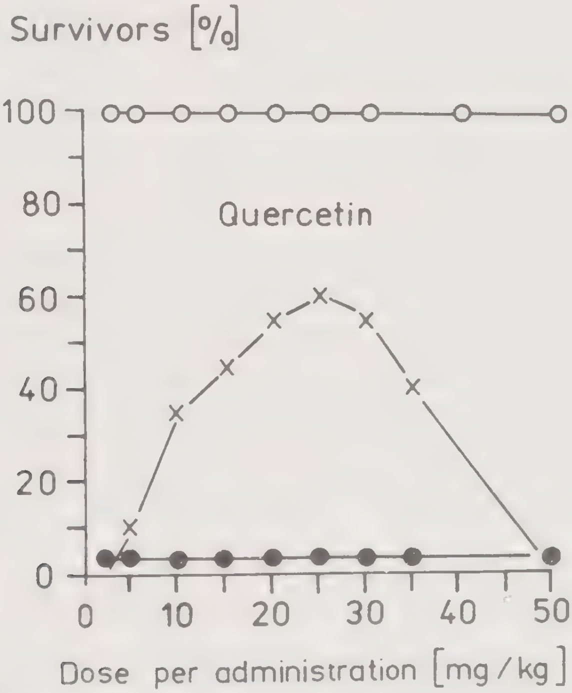

Fig. 5. Dose-response curves of quercetin (20 mg/kg body mass per administration, p.o.) in both non-infected and Mengo virus-infected (10 LD_{50}, i.p.) $ABD2F_1$ mice. Treatments were performed at hours -1, +12, +24, +32, +48, +56, +72 and +80, relative to virus inoculation. Drug-treated and sham-infected (o); drug-treated and infected (x); placebo-treated and infected (●).

However, quercetin caused strikingly different effect in Mengo virus-infected mice (10 LD_{50}, i.p. or i.nas.). Animals were protected from lethal infection in a dose-dependent manner, but when given in abundance, the drug exerted a decreased protection (Fig. 5). This effect has been found to be independent of the murine genotype, although sensitivity to the antiviral activity of quercetin varied between the mouse strains used. Thus, it is suggested that high doses of the drug diminish the animals' defence to the virus infection (29).

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ANIMAL DISTRIBUTION AND PRELIMINARY HUMAN KINETIC STUDIES OF THE FLAVONOID FRACTION OF A STANDARDIZED *GINKGO BILOBA* EXTRACT (GBE 761)

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INTRODUCTION

The Ginkgo biloba extract is a specific and complex product prepared from the dry green leaves of Ginkgo biloba (GB 761).

The leaves are harvested from trees growing in plantations in South Korea and Japan and, since last year, in France. The crude extract is purified and enriched during an extraction process consisting of fifteen different steps, which leads to a precisely standardized extract.

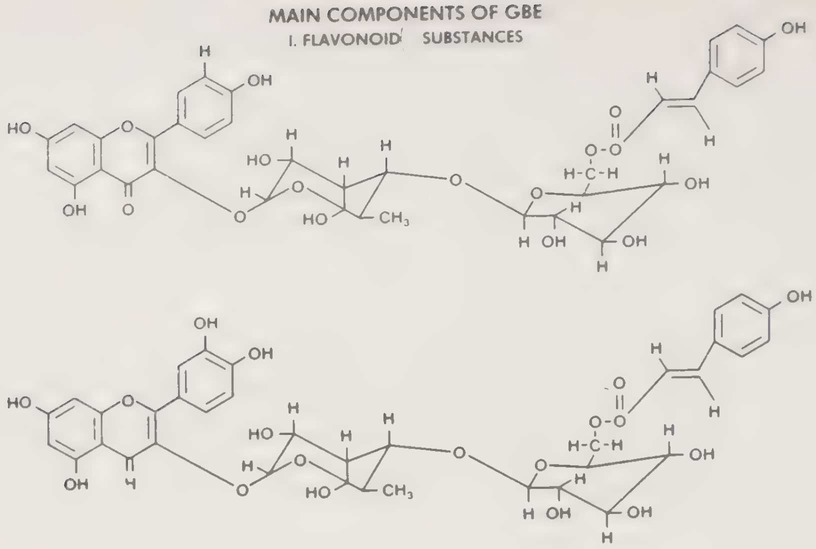
It consists of about 40 % of flavonoid substrances: proanthocyanidins and kaempferol, quercetine and isorhamnetine glycosides, some of them being specific to Ginkgo (Figure 1).

The non-flavonoid fraction of the extract consists of organic acids and terpenoids which are characteristic of Ginkgo, namely ginkgolides and bilobalide.

The extract is standardized on 24 % content of Ginkgo flavone glycosides with a reproducible pattern of these glycosides (Fig. 2). More than 20 peaks were identified.

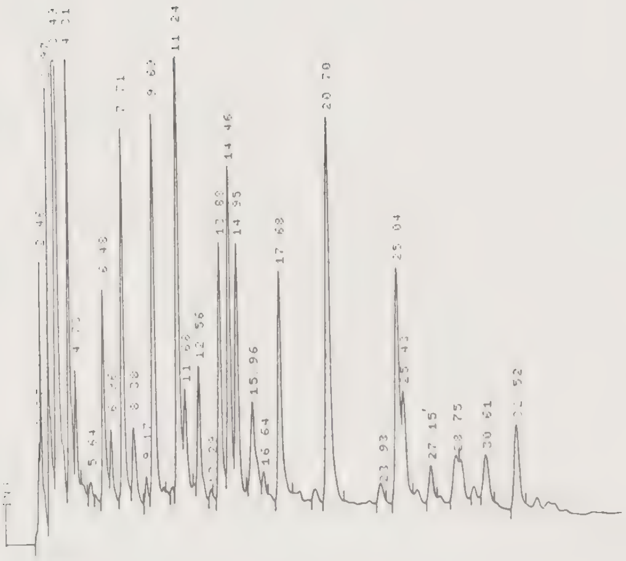
Ginkgo biloba extract is marketed in France, FRG, Spain, Italy, Greece and a few more countries and it has been widely prescribed over the last 10 years. It is used in organic brain impairment in aging, in auditory and visual deficiencies and in peripheric vascular disorders.

Much pharmacological work has been performed on Ginkgo biloba extract. Although the effects are shared between the different constituents, the pharmacological properties of



MAIN COMPONENTS OF GBE
I. FLAVONOID SUBSTANCES
HO
OH
O
H
HO
H
HO
CH$_3$
O
O
O
H-C-H
O-O
OH
H OH
OH
OH
H

Fig. 1.



3 46
4 31
6 48
7 71
9 60
11 24
5 64
8 38
9 17
11 88
12 56
13 80
14 46
14 95
15 96
16 64
17 68
20 70
23 93
25 04
25 43
27 15
28 75
30 61
31 52

Fig. 2.

flavonoids play a large role in the observed effects. They have been shown to be effective against increased capillary permeability, to protect the blood-brain barrier (4), to improve brain energy metabolism (5-7) and to prevent edema (5-6). They also influence the regulation of blood flow in small vessels whether it has to be reinforced or limited (8). Ginkgo biloba extract has also free radical scavenging properties (9) (J. PINCEMAIL: this symposium).

Very few data on flavonoids bioavailability and kinetics are available (10-11); but it was generally assumed that after oral administration they are not absorbed before degradation by the intestinal gut (12). However, many factors may affect their bioavailability, and we wanted to know if their chemical environment in GBE plays a role in their absorption. Thus, studies in rats and humans were undertaken to determine the biological fate of the flavonoid constituents of GBE administered orally.

1. METABOLIC AND KINETIC STUDIES

We initiated our metabolic studies by oral administation of a large dose of GBE in rats. Early studies were essentially qualitative and allowed to identify the following metabolites, essentially as glycuroconjugates (in bile and urine):

- aglycones, i.e.: kaempferol, quercetin and isorhamnetin
- phenolic acids: hydroxyphenylacetic - hydroxybenzoic - hydroxyphenylpropionic - vanillic - homovanillic and coumaric acids. Secondly, we performed in rats urine a quantitative determination of phenolic acids by gas chromatography/mass spectrometry, but even with a flavonoid-free diet, only vanillic acid exhibited a consistent response to GBE treatment.

Thus it became apparent that the monitoring of flavanoids after enzymatic hydrolysis would be more suitable for further pharmacokinetic studies.

We developed a very sensitive assay be a HPLC/electrochemical detection method to measure aglycones in urine and blood (sensitivity: 0.5 µg/ml); this method was sensitive enough to consider the possibility to analyse kaempferol and

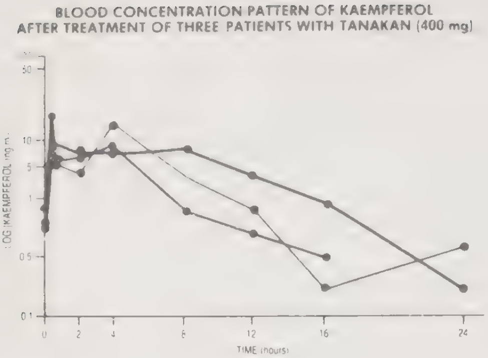


Fig. 3.

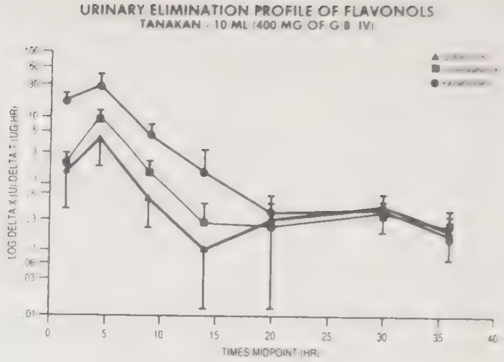


Fig. 4.

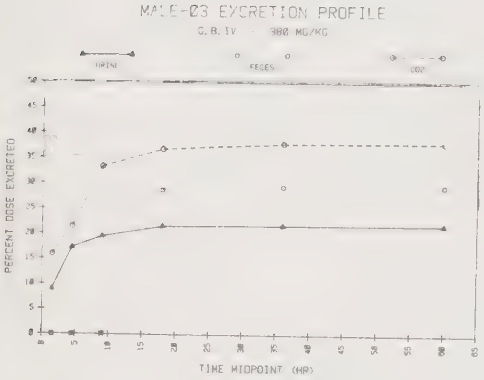


Fig. 5.

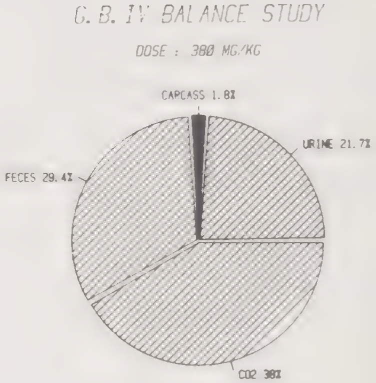


Fig. 6.

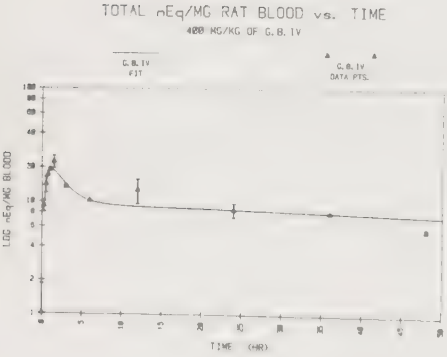


Fig. 7.

quercetin in human blood and urine after administration of reasonable doses of GBE.

A 400-mg dose of GBE corresponding to 3 times the therapeutic daily dose was given to volunteers previously kept for 3 days under a flavonoid-free diet. Blood samples were taken at 30 min - 1 - 2 - 4 - 8 - 16 and 24 hours, and urine collected at sampling intervals of 0-4, 4-8, 8-16, 16-24, 24-36 h. A preliminary study was done in 3 subjects. The blood concentrations are given in Fig. 3.

Although one can observe a very large inter-individual variation, the kaempferol concentration profile shows 2 maxima suggesting 2 sites of absorption. The most unexpected is certainly the fact that the first peak occurs within 30 minutes after oral administration.

We know that the ginkgo-flavone-glycosides are not cleaved by gastric juice, so it can be assumed that they are absorbed as glycosides.

The second peak at 4 hours may represent the intestinal absorption of the remaining substances after exposure to the colonic bacteria.

The calculated half-life is about 3 hours. The urinary excretion profile (Fig. 4) of kaempferol, quercetin and isorhamnetin follows parallel courses in relationship with their relative abundance. A maximum occurs around 4 to 5 hours post-dose, suggesting again that the biological disposition of the flavonols of GBE is rapid. By 20 hours the urinary excretion is almost completed. These data suggest that the "environment" of flavonoids in GBE may enhance their bioavailability.

2. DISTRIBUTION STUDY IN RATS

The distribution of a ^{14}C labelled GBE in rats was examined. Twigs of young Ginkgo tree were fed in a phytotron with a solution of ^{14}C-acetate at the University of Heidelberg. The leaves were dried and extracted and the extract was purified in a similar way to the one used for industrial purpose.

The product exhibited a specific radioactivity of 0.23 µCi/mg.

An HPLC analysis of the extract showed that its flavonoid part was almost exclusively labelled on ring A, which arises from the biosynthetic pathway through acetate. Components of the extract which should not be derived from the acetate pathway were not labelled, and the sugars of the heterosides which arise from the photosynthetic pathway were not labelled. Sprague-Dawley rats, male and female, received 20 µCi of GBE per animal (a dose of 400 mg/kg was necessary). Blood samples were taken at different time intervals; urine and feces were collected and CO_2 trapped. Three groups of rats were killed at 3-48 and 72 hours. Tissue samples were then collected and radioactivity was assayed by liquid scintillation counting. $^{14}C\text{-}CO_2$ represents the major part of the radioactivity eliminated in the first 3 hours and was still exhaled until 12 hours. In urine, the elimination rate constant corresponds to a half-life of 7 hours (Fig. 5). Most of the radioactivity associated with feces is eliminated between 12 and 24 hours (Fig. 5).

Thus the bioavailability of the drug was greater than 60 % based on the radioactivity balance at 72 hours, which shows 38 % exhaled in CO_2, 22 % excreted in urine and early 2 % remaining in the body (Fig. 6).

The blood pharmacokinetic of total nanoequivalents of ^{14}C-GBE is shown Fig. 7.

It fits with a 2 compartments model with a biological half-life of 1.2 hours for the first compartment, and a very long half-life for the second, expressed by the almost horizontal slope of the terminal part of the curve.

The tissue distribution has been investigated at 3-48 and 72 hours. The elimination from the gastrointestinal tract (Fig. 8) is faster than in the plasma; carcass and kidney have an elimination rate similar to that of plasma; it is slower in the liver (Fig. 9).

As the brain is concerned (Fig. 10), the radioactivity in cortex, midbrain, cerebellum and brain-stem is smaller than in plasma. However, in the hippocampus, striatum and hypothalamus the radioactivity is much higher and especially at 72 hours: at

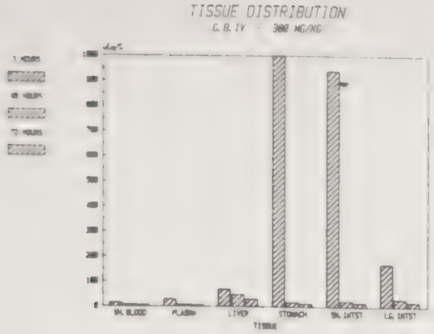


Fig. 8.

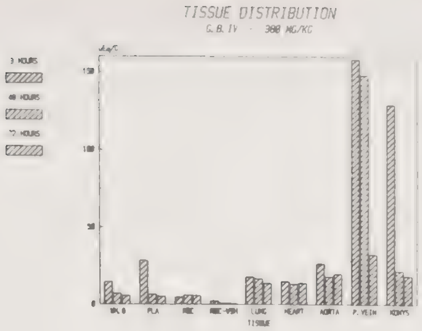


Fig. 9.

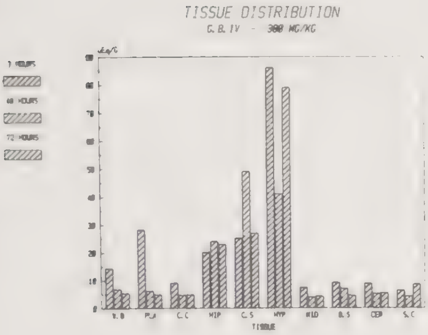


Fig. 10.

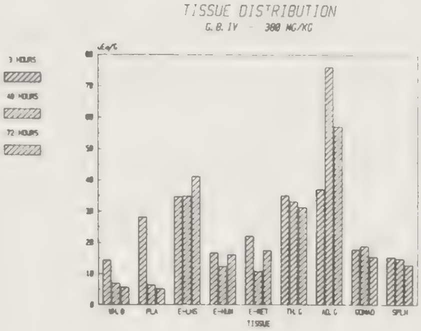


Fig. 11.

that time the remaining radioactivity is, respectively, 5.5- and 16-fold the one of plasma.

In addition to these results (Fig. 11) is was noticed that the radioactivity was also retained in eye lens, thyroid and adrenals (8-6- and 15-fold the plasma level).

Thus it can be assumed that the results are due to the fixation of flavonoids or of their metabolites. The significance of this affinity at the level of neuroendocrine tissues is under investigation, and we already know that a regulation of blood flow and brain energy metabolism in ischemic models is seen in hypothalamus, hippocampus and striatum under Ginkgo biloba treatment.

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INFLUENCE OF THE FLAVANOLIGNAN DERIVATIVE SILIBININ ON NUCLEIC ACID AND PROTEIN SYNTHESIS IN LIVER CELLS

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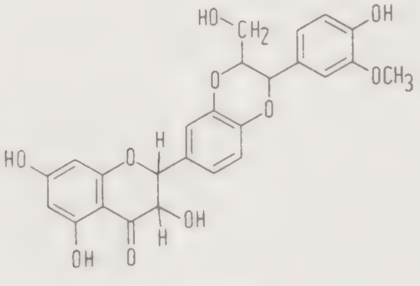
Extracts of the plant Silybum marianum have been used for centuries in cases of liver disease for a better recovery. In 1965 Wagner and Hörhammer and also Hänsel and Pelter isolated the flavonolignan derivative Silibinin as the active drug.



Silibinin

In this lecture I would like to give data concerning the biochemical action of this flavanolignan derivative on the metabolism of liver cells. It will be shown that Silibinin primarily stimulates RNA synthesis in hepatocytes and in isolated cell organelles, but also in rat livers. In order to explain the methods which have been used to follow nucleic acid synthesis I would like to remind you on the basic process of gene transcription and translation, Fig. 1.

In eukaryotic cells the DNA is transcribed by the DNA-dependent RNA-polymerases I, II and III to different RNA types. Polymerase I is competent for the transcription of the ribosomal RNAs which are constituents of the ribosomes. Polymerase II synthesizes the messenger RNAs which bear the genetic informa-

TRANSCRIPTION

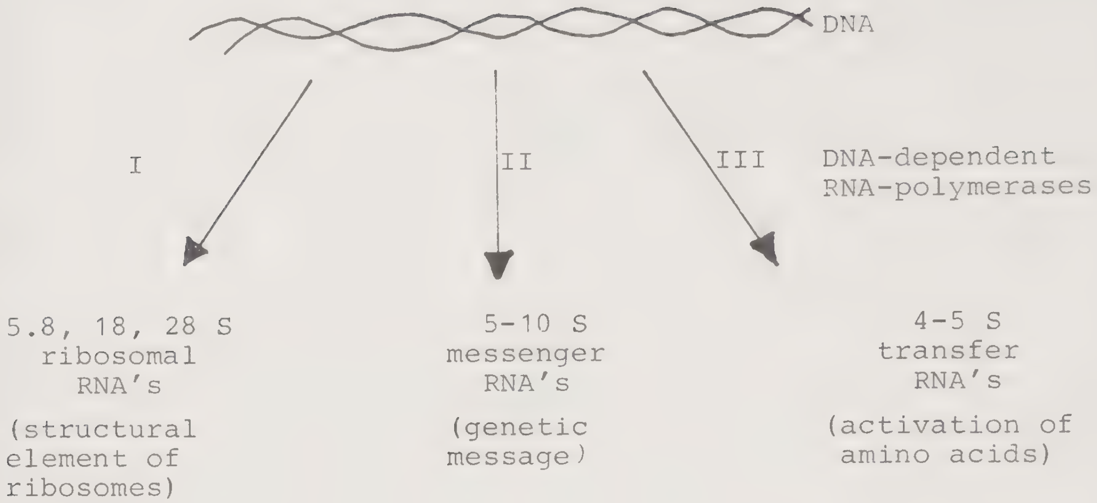

DNA
I
II
III
DNA-dependent RNA-polymerases
5.8, 18, 28 S ribosomal RNA's
(structural element of ribosomes)
5-10 S messenger RNA's
(genetic message)
4-5 S transfer RNA's
(activation of amino acids)

Fig. 1.

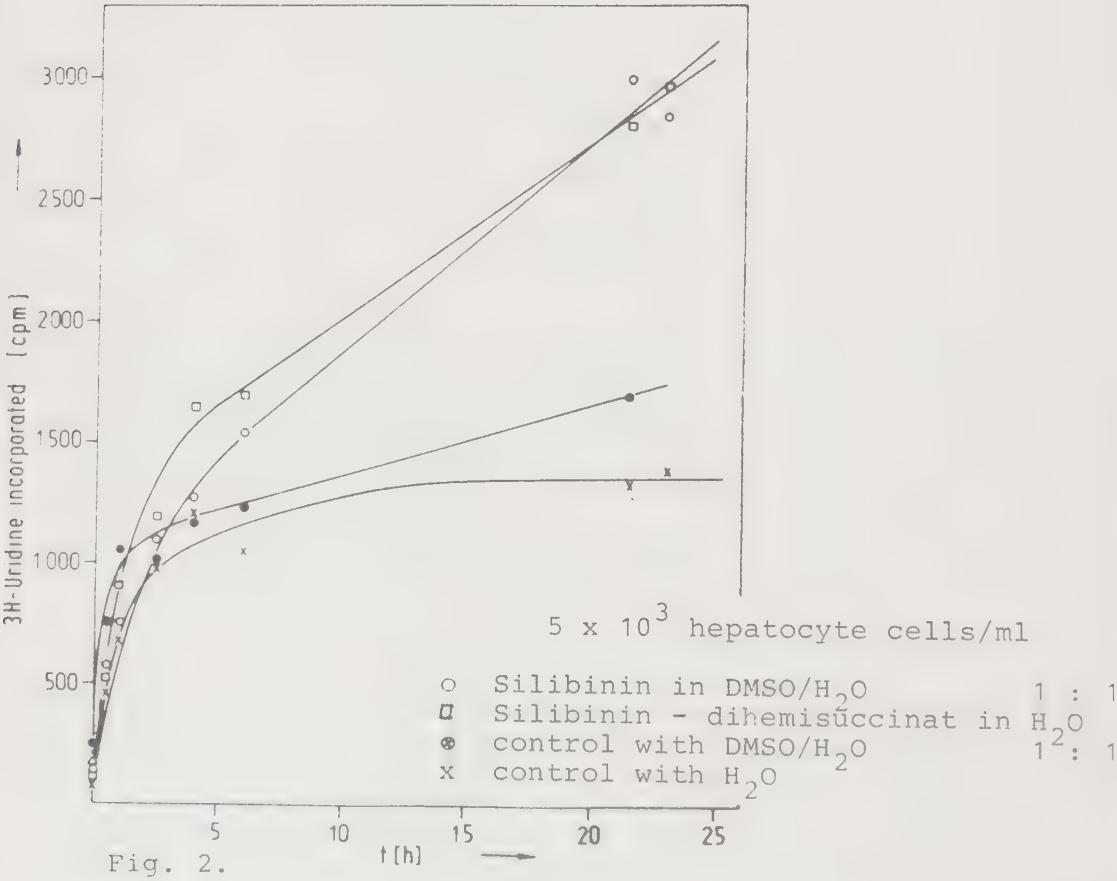


Fig. 2.

tion, and polymerase III forms the small tRNAs which are necessary to activate the amino acids during the translation process of protein synthesis. One specific constituent of all RNA species is uridine, which can be used with a radioactive label as a precursor and an indicator for RNA synthesis.

Hepatocytes in culture are able to proceed in RNA synthesis. With tritium-labelled uridine as one of the nucleotide precursors, we can measure the time-dependent course of RNA formation. It was a fascinating moment when we found that in the presence of small Silibinin concentrations the RNA synthesis in hepatocytes is stimulated, Fig. 2.

Optimal stimulation effects can be seen with 2-10 µg Silibinin per ml medium. In this range an increase of RNA synthesis by about 25 % is observed. With much higher concentrations, however, also "inhibitory effects" can be seen, as the consequence of a reduced precursor penetration through the cell membranes, which might be caused by unspecific interactions of the polyphenol with membrane proteins, thus influencing permeabilities.

In view of pharmacological aspects, it was very interesting if the stimulation in RNA synthesis with low Silibinin concentrations can also be observed with rats in vivo. We investigated, therefore, in a series of experiments with rats the RNA synthesis in vivo and measured the dose- and time-dependence of uridine incorporation after intraperitoneal application of Silibinin. As a precursor, instead of uridine, radioactive orotic acid was given. After definite times the livers were resected and the RNA was isolated. For these in vivo experiments the Silibinin hemisuccinate ester was used, because the ester has a much better solubility in water. Koch and Tscherny have described that the succinic esters are split in the tissues by esterases within a few minutes into free Silibinin. Parallel to the analysis of the specific radioactivities of the RNA, it had to be checked that the pools of radioactive precursor are the same in the livers with and without treatment of Silibinin, otherwise differences in the specific radiactivity of the newly synthesized RNA would be a consequence.

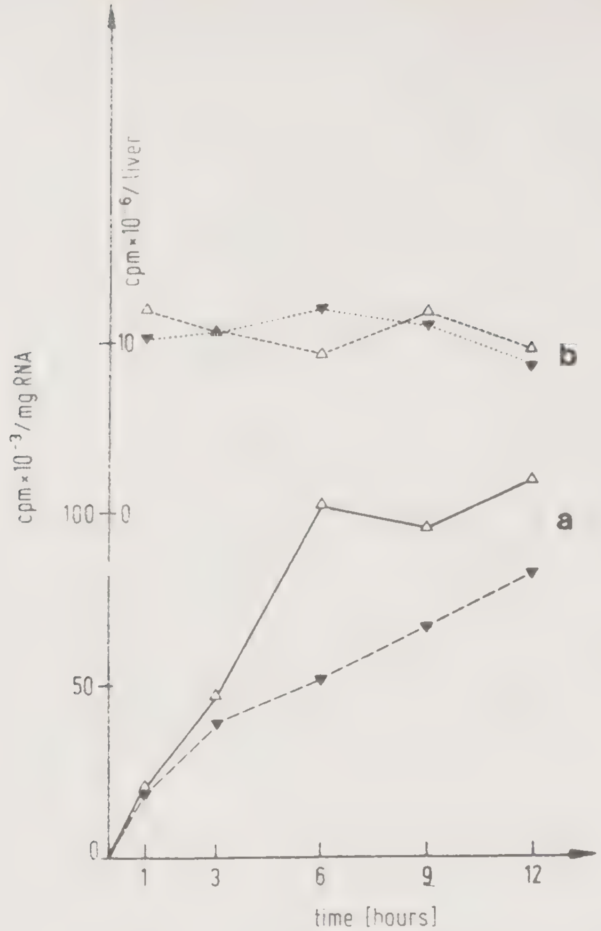


Fig. 3. a) specific radioactivities of liver RNA
b) precursor concentrations cpm/g liver
△ after treatment with Silibinin
▲ controls

From Fig. 3 can be seen that the precursor pools in the livers are quite similar and independent of the application of small doses of Silibinin. In the presence of the flavanolignan the rate of RNA synthesis, however, is significantly higher than in the controls. In the experiments a maximum stimulation effect can be observed with about 25 mg Silibinin per kg rat.

The question had to be asked if the increase in RNA synthesis is a general one concerning all RNA species, or if this stimulatory effect is specific, for instance affecting only the mRNA or tRNA, etc. Therefore, after isolation the RNAs species were fractionated and their specific radioactivities analyzed.

Table 1. Specific radioactivities [cpm/mg] of RNAs from Silibinin treated rats and controls

a) ribosomal RNA

	total RNA	28S RNA (43 %)	18S RNA (23 %)
treated with Silibinin	138800	83250	74000
controls	69700	64750	42310

b) small RNAs

	cytoplasmic RNA	tRNA (7.2 %)	5 S-RNA (1.4 %)	5.8 S-RNA (1.2 %)
treated with Silibinin	282000	105560	91220	67230
controls	223000	104260	89050	50410

c) m-RNA

	cytoplasmic RNA	nuclear RNA	mRNA (cytoplasm) (1.3 %)	mRNA (nuclei) (0.7-2 %)
treated with Silibinin	282000	626000	908900	68500 (2 %)
controls	223000	402200	1042000	73900 (0.7 %)

Electrophoreses on agarose gels and sucrose gradient centrifugations were used. From Table 1 can be seen that the specific radiactivities of the RNA from Silibinin-treated and untreated rats are quite similar for the messenger RNAs, the small tRNAs and the 5 S RNA, but remarkably different for the 5.8 S RNA, 18 S RNA and 28 S RNA. We also find great difference for the 45 S RNA species which is not listed in the Table but which represents the precursor molecule for the 5.8, 18S and 28S RNAs. The data show that Silibinin specifically stimulates the transcription rate of ribosomal RNA.

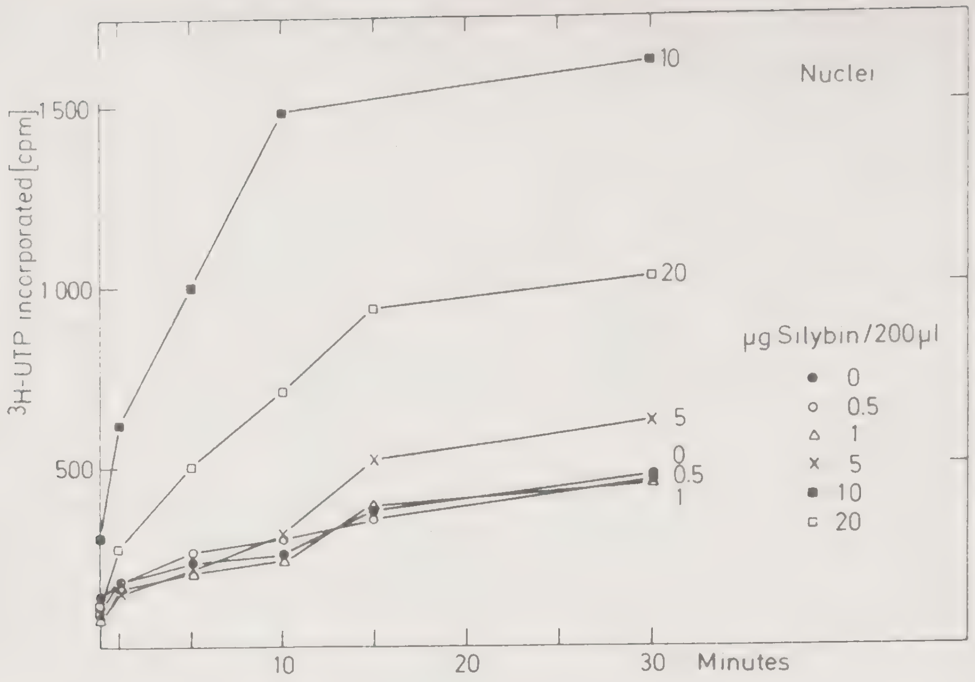


Fig. 4.

In order to elucidate the molecular mechanism of this effect, a series of experiments was done with isolated nuclei and also nucleoli which still have the ability to synthesize RNA *in vitro*. In Figure 4 the time-dependent rate of RNA synthesis in isolated nuclei can be seen in the presence of different Silibinin concentrations.

A maximum of stimulatory effect can be seen with about 50 µg Silibinin per ml.

To elucidate the molecular mechanism of the effect we had to further simplify our experimental system. In the following experiments the isolated RNA polymerases I and II were used with pure DNA from calf thymus as a template in buffer with the nucleotide triphosphate, uridine labelled by tritium, Fig. 5.

With polymerase II, which can be inhibited by amanitin and which is competent for the production of messenger RNA, we could not find any effect of Silibinin on the time course of transcription. With polymerase I, which is not sensitive to amanitin and which is competent for the transcription of the

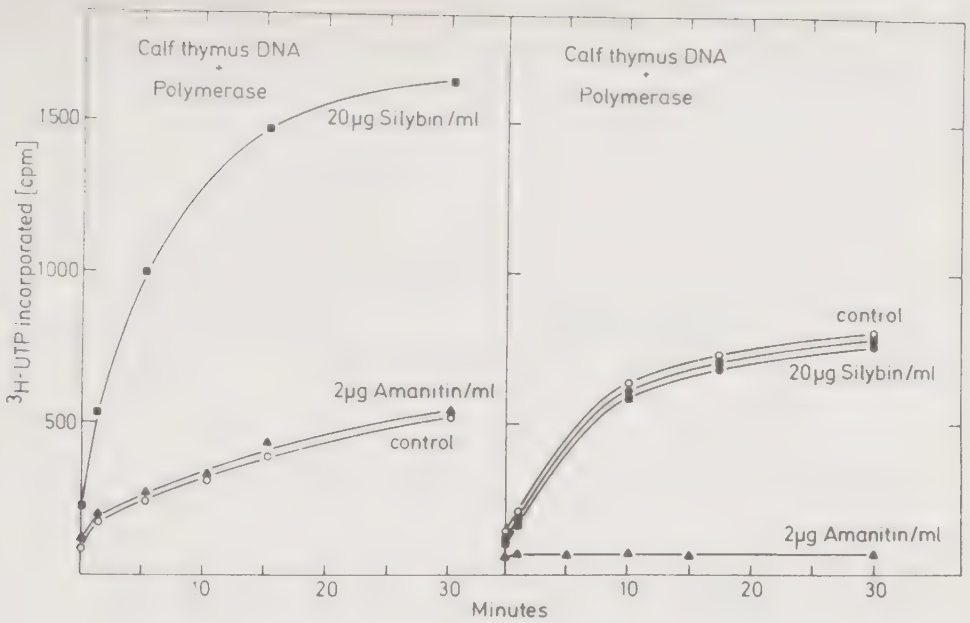


Fig. 5.

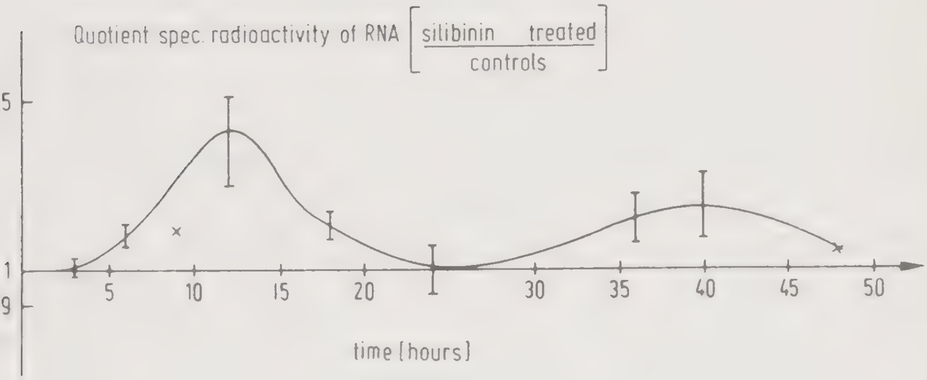
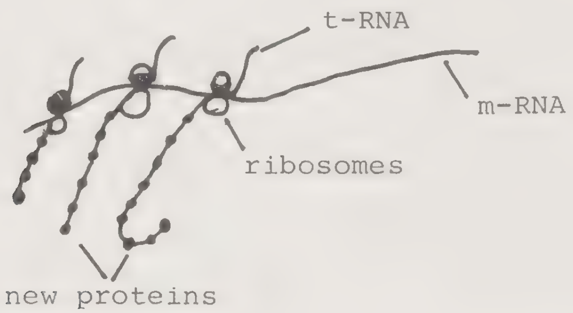


Fig. 6.

ribosomal genes, a remarkable increase in the rate of synthesis was found.

This result is in good agreement with our findings from the *in vivo* experiments. In both experimental approaches we observe an effect of Silibinin on the synthesis of ribosomal RNA. From the experiments with the purified polymerases we can say that the enzyme is activated by the drug comparable to the action of some enzyme effectors. By further experiments with isolated polymerases we could demonstrate that specifically the elongation rate during transcription is influenced and increased by the flavanolignan derivative.

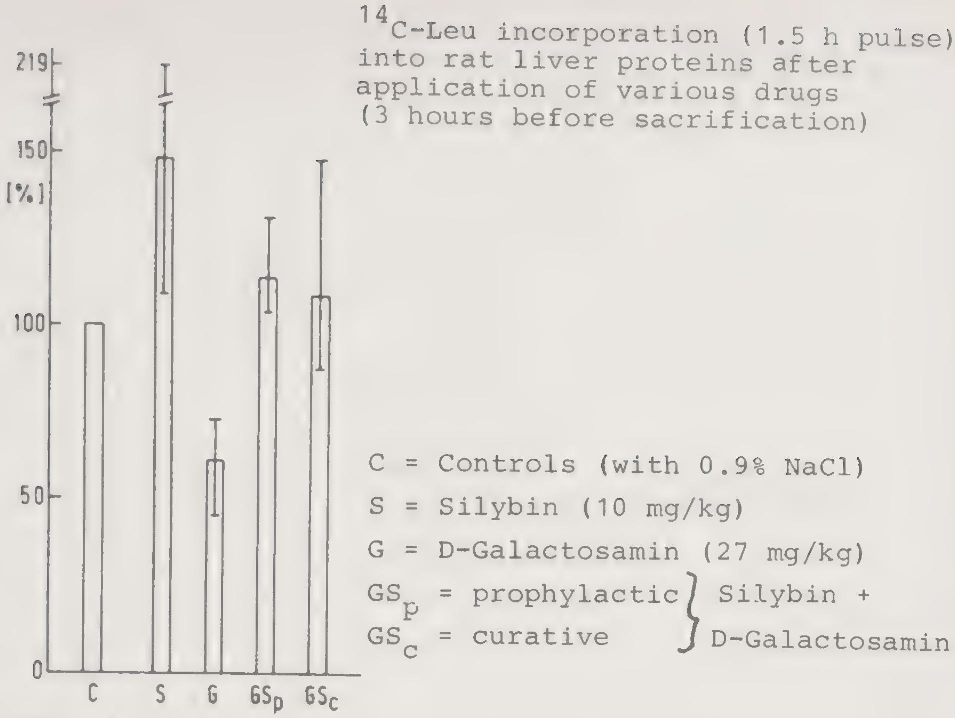


Ribosomes are built from the ribosomal RNA's together with about 84 proteins and can be seen as the sewing machinery within the cytoplasma for protein synthesis. Does the increase of rRNA synthesis by Silibinin imply also a stimulation of ribosome formation? In order to answer this question we isolated the ribosomes of rat livers after in vivo labelling of the RNA by tritium-uridine and after simultaneous labelling of the ribosomal proteins with ^{14}C-leucin or isoleucin. The stimulatory effect of Silibinin also on the synthesis of mature ribosomes can be seen in Fig. 6.

The increase in ribosome formation is plotted against the time of application of the drug. One can see that three are two waves of response, one maximum about 12 h after application of Silibinin and the second at about 35 h.

If the rate of synthesis of ribosomes is stimulated, the sewing machinery for translation is increased, therefore, also effects on protein synthesis might be expected. To investigate this hypothesis, we measured both protein synthesis in hepatocytes in vitro and also in rat livers in vivo. ^{35}S-methionine or ^{14}C-leucin were used as precursors, alternatively.

From Fig. 7 can be seen that, indeed, protein synthesis follows the increase in rRNA transcription and ribosome formation. We investigated if there occur specific effects on definite polypeptides. The proteins were isolated and fractionated



^{14}C-Leu incorporation (1.5 h pulse) into rat liver proteins after application of various drugs (3 hours before sacrification)

C = Controls (with 0.9% NaCl)
S = Silybin (10 mg/kg)
G = D-Galactosamin (27 mg/kg)
GS_p = prophylactic } Silybin +
GS_c = curative } D-Galactosamin

Fig. 7.

in one-dimensional and two-dimensional electrophorectic systems with autoradiographic detection. No differences in the pattern and the label of distinct proteins could be found. The conclusion is drawn that, as a secondary effect of RNA stimulation, also protein synthesis is increased but without preference of specific proteins.

From clinical findings it is known that Silibinin supports the regeneration activity of liver tissue after intoxication (Fintelmann and Albert). From the biochemical point of view regeneration activity means proliferation and DNA synthesis. The already described results demonstrate that Silibinin activates the RNA polymerase I and as a consequence stimulates ribosomal RNA synthesis, ribosome formation and stimulates protein synthesis. Protein synthesis and RNA synthesis are prerequisites for DNA synthesis. So it seemed reasonable that also DNA synthesis is influenced by the flavanolignan derivative. To measure DNA synthesis, radioactive thymidine is given as a precursor. With several cell lines in culture, however, we could not detect any effect of Silibinin on the rate of proliferation or

Table 2. DNA-synthesis in a) normal livers, b) after partial hepatectomy (68 %), with and without Silibinin treatment

Silibinin-2′, 3-dihydrogensuccinate sodium salt [mg/kg body weight]	Number of rats	10^{-3} x dpm/mg DNA [mean values and standard deviations]	Precursor pool: Radioactivity 10^{-3} x dpm/1 g liver tissue (containing about 3 mg DNA)
a) 0 (controls)	16	105 ± 12	1109 ± 18
17	3	127 ± 26	1017 ± 27
30	10	99 ± 9	1210 ± 9
50	3	118 ± 16	1314 ± 41
b) 0 (controls)	10	1922 ± 287	10285 ± 1003
0	10	1921 ± 338	10339 ± 1636
27	10	2367 ± 326	10261 ± 1846
27	10	2306 ± 290	9343 ± 1304

on the rate of DNA synthesis. This was also the case if we measured the DNA synthesis in livers of normal rats. But in livers of hepatectomized rats, an effect of Silibinin on DNA synthesis could be observed.

From Table 2 can be seen that after hepatectomy the rate of DNA synthesis in liver cells increases dramatically. Moreover, the data show that the increase after treatment with Silibinin is still greater. This means that if the trigger for DNA synthesis and proliferation once is given, the stimulation of RNA and protein synthesis by Silibinin also increases the rate of DNA synthesis. This confirms the clinical finding of a better regeneration potency of damaged livers in the presence of the flavanolignan derivative.

Fig. 8 summarizes the results.

The described effect of Silibinin on such a fundamental process as transcription is fascinating because here we find the action of a plant drug on an animal enzyme system. The question arose what part of the Silibinin molecule might be responsible for the effect and if there is any relationship to known enzyme effectors in animal cells so that receptor sites can be occupied competitively. In transcription systems with nuclei and hepatocytes the influence of about 40 flavonoids and

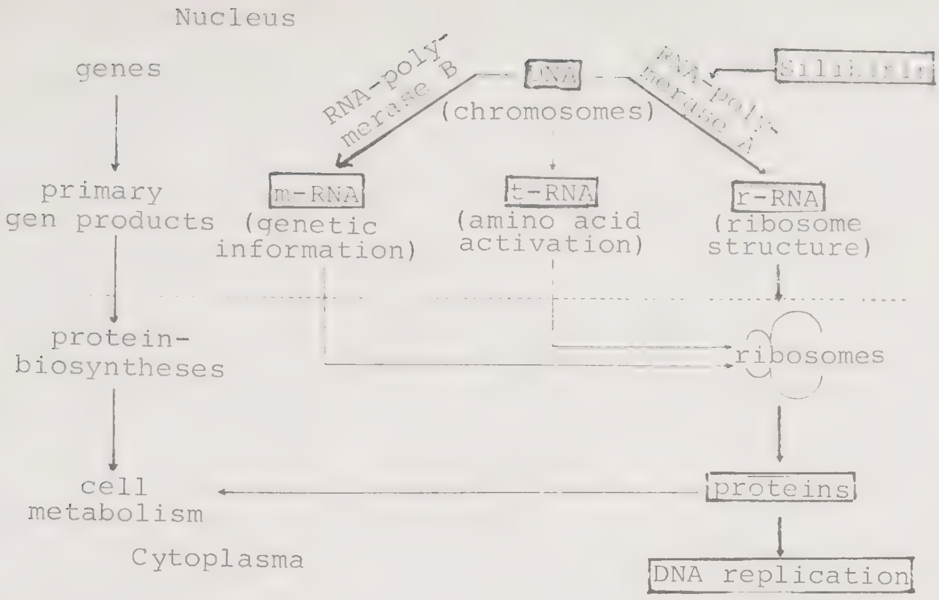


Fig. 8.

flavanolignan compounds on RNA synthesis was investigated. Only four of these compounds showed an effect similar to that of Silibinin. We tried to elaborate the structures which seem to be essential. From this the speculation is drawn that there exists a relationship to steroid hormones which are well known effectors in the regulation of animal cells and which show time response curves also on polymerase A which are quite similar to those for Silibinin. So I would like to finish this lecture with the speculation that Silibinin imitates in some way a physiological regulator in animal cells, that the structure might fit into a specific binding site on the polymerase and in such a way causes the observed effects on rRNA synthesis making the drug from Silybum marianum interesting for liver therapy.

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THE EFFECTS OF FLAVONOIDS ON HUMAN PLATELET FUNCTION

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INTRODUCTION

Platelet interactions with surfaces are important events in the initiation of thrombosis and atherosclerosis. Pharmacological inhibition of platelet function is useful in the treatment of thrombotic diseases /1,2/. Flavonoids have been shown to inhibit some aspects of platelet function. These results were obtained using platelets from various animal species, resuspended in plasma or washed, and a wide variety of flavonoid types. In this review, we will summarize the results concerning the effects of flavonoids on platelet adhesion and aggregation, in vitro and in vivo. From these studies it is possible to draw some conclusions from structure-activity relationships and to discuss possible mechanisms responsible for the actions of flavonoids on blood platelets.

EFFECTS OF FLAVONOIDS ON PLATELET ADHESION

Adhesion is the initial event in the interaction of platelets with foreign surfaces such as collagen and other extracellular macromolecules of the subendothelium, damaged endothelial cells, smooth muscle cells of the media and artificial surfaces of biomaterials in contact with blood /3/. For example the inhibition of platelet adhesion should be of therapeutic value in the prevention of thrombosis and of the thromboembolic complications of atherosclerosis and extracorporeal circulation. Flavonoids have been shown to protect in vivo the endothelial lining of the vessel wall /4,5,6/. Flavonoids decrease the adhesion of platelets to glass wool filters /7/ and to collagen-coated surfaces /8/. These results show that compounds with a low polarity are better inhibitors of adhesion (importance of methyl groups /7/, stronger activity of flavone compared to hydroxylated molecules /8/), while the presence of the C2, C3 double bond has no influence /8/. These structural requirements are different from those described for inhibition of platelet aggregation and secretion by flavonoids. This suggests that different mechanisms are operating in order to inhibit adhesion to foreign surfaces rather than platelet to platelet adhesion, that is platelet aggregation.

EFFECTS OF FLAVONOIDS ON PLATELET AGGREGATION AND SECRETION

The possibility that flavonoids could inhibit platelet function was mentioned by investigators studying O-(β-hydroxyethyl) rutosides (HR), a complex mixture of semisynthetic derivatives of rutin present in commercial drugs. HR (0.5 to 5 mM) inhibited the aggregation of human or rat platelets in citrated platelet-rich plasma (PRP) induced by adrenaline /9/ or ADP /9,10,11/. The administration of HR (500 mg/kg subcutaneously for 3 days /10/ or 100 mg/kg per os for 22 days /11/) to rats, decreased the sensitivity of platelet aggregation to ADP. A double blind crossover study in man showed that oral administration of HR (3 g/day) slightly reduced the secretion of serotonin (5-HT) from platelets, but had no detectable effect on collagen or ADP-induced platelet aggregation /12/. Intravenous injection of 1.5 g HR to human volunteers did not significantly inhibit platelet aggregation by ADP or collagen and the production of malondialdehyde, an oxidation product of arachidonate /12/. Intravenous administration of HR to rats subjected to endothelial damage decreased the number of circulating endothelial cells and reduced the interaction of platelets with the damage vessel wall, but had no effect in preventing venous or arterial thrombosis /5/.

Natural flavonoids are also inhibitors of platelet function. They inhibit the aggregation of human /8,13,14,15,16,17,18/, bovine /19/ or rabbit /20/ platelets. Quercetin and other flavonoids inhibit the secretion of 5-HT induced by collagen or thrombin /14/ and by tannin /19/. The best inhibitors are active in the 10 μM range. These effects of flavonoids have been studied with PRP or washed platelets. However, Moore et al. /21/ have not been able to find any effect of rutin, naringenin or quercetin on ADP-induced aggregation in human PRP. We have prepared /14,22/ PRP and washed platelets from the same donor and compared the effect of quercetin and flavone on platelet aggregation. Table 1 shows that quercetin inhibits

Table 1 : Comparison of the effect of flavonoids on the aggregation of human platelet-rich plasma or washed human platelets from the same donor.

Flavonoid	Platelets	Aggregating agent	
		A D P (5 µM)	Collagen (2.5 µg/ml)
Quercetin	PRP	6 % inhibition of 1st wave at 200 µM 51% inhibition of 2nd wave at 200 µM	No inhibition at 200 µM
	Washed	IC_{50} = 64 µM	IC_{50} = 39 µM
Flavone	PRP	10% inhibition of 1st wave at 200 µM 57% inhibition of 2nd wave up to 10 µM	IC = 66 µM
	Washed	IC_{50} = 54 µM	IC_{50} = 18 µM

aggregation when platelets are washed and resuspended in Tyrode's solution containing a low (3.5 mg/ml) concentration of albumin. In contrast, when platelets are suspended in PRP, which contains a high concentration of plasma proteins, quercetin has a marginal inhibitory effect on platelet aggregation. Similarly, the protein concentration in the suspending medium influences the inhibitory effect of flavone on platelet aggregation induced by ADP or collagen.

STRUCTURE-ACTIVITY RELATIONSHIPS

The study of the inhibition of platelet aggregation by drugs is a useful means to compare the pharmacological potency of inhibitors /22/ and to deduce structure-activity relationships in intact cells. From inhibition studies of aggregation of washed human platelets, we have pointed out some of the essential structural requirements for a flavonoid to inhibit platelet aggregation /14/ : a) glycosides are much less effective than the corresponding aglycones; b) the presence of the C2, C3 double bond is essential; c) a high number of OH group can decrease the activity.

A number of these structure-activity relationships required for inhibiting the function of intact platelets are similar to those found for inhibiting isolated enzyme preparations, such as cAMP phosphodiesterase (PDE) /23/ or cyclooxygenase /17/.

There are other compounds related to flavonoids that can inhibit platelet aggregation : chalcones /14,17,20/, substituted flavanones /20/, pterocarpans /20/ or substituted benzopyranones /24/.

Flavonoids inhibit the aggregation of platelets induced by a wide variety of agonists : ADP /9,10,11,13,14,15,16,17,20/, collagen /13,14,16,17,20/, adrenaline /9,16/, thrombin /14/, phorbol ester /18/, tannin /19/, calcium ionophore A 23187 /13/, arachidonic acid /16,17,20/. These agents act through at least three different mechanisms /1,2,22/. If flavonoids inhibit the effects of all types of platelet activators through the same mechanism, this must be due to inhibition of a late step in the activation process, such as an effect on cytoplasmic Ca2+ levels. A rise in intracellular cAMP, which can be achieved either through stimulation of membrane adenylate cyclase and/or through inhibition of cAMP phosphodiesterase (PDE), is followed by a reduction in the concentration of cytoplasmic Ca2+ and results in the inhibition of platelet function /1,2/. The evidence that PDE inhibition plays a role in the inhibition of platelets function by flavonoids is as follow : a) flavonoids are potent inhibitors of PDE preparations /23,25,26,27/ ; b) structure/activity relationships are similar for PDE inhibition /23,25,27/ and for platelet anti-aggregatory action of flavonoids /14/; c) quercetin induces a rise in platelet cAMP in the presence of PGI_2 or PGE_1 /15,17/. However, it has also been shown that some compounds (flavone, flavanone) did not cause a rise in platelet cAMP and could even antagonize the effect of PGI_2 on cAMP levels in human platelets /16,17/.

Flavonoids can also inhibit the action of a specific agonist. For example, flavonoids inhibit cyclooxygenase, an enzyme complex involved in the oxidative transformation of arachidonic acid into thromboxane A_2, a potent aggregatory and vasoconstrictive eicosanoid /16,17,28,29,30/. There is strong evidence that the inhibitory effect of flavone on platelets is mainly due to inhibition of cyclooxygenase /16/. These effects of flavonoids on prostaglandin metabolism could also be a consequence of the rise in cAMP caused by PDE inhibition /17/. However, the inhibition of cyclooxygenase alone cannot be the mechanism by which flavonoids inhibit the aggregation of washed human platelets caused by ADP, thrombin or ionophore A 23187, because these stimuli activate platelets independently of the formation of thromboxane A_2 /22/.

There are other biochemical properties of flavonoids which could play a role in their mechanisms of action on blood platelets. Flavonoids inhibit another enzyme of the arachidonate cascade, a lipoxygenase which is involved in the transformation of arachidonic acid into chemotactic hydroxyperoxides /29,30,31,32,33/. Hydroxyethyl rutosides (HR) inhibit the uptake of adenosine by red cells and platelets /9/; the subsequent rise in plasma adenosine could, through adenylate cyclase stimulation in platelets, have an anti-aggregatory effect. Other hypothetical mechanisms of action of flavonoids on platelets include the inhibition of a membrane-associated NADH-dependent cytochrome C reductase /13/, the regulation of glycolytic activity /10/, the scavenging of free radicals /30/ or a non-specific effect at the platelet surface /8,9/.

DESIGNING A FLAVONOID WITH ANTIPLATELET EFFECT

In search for new active compounds, we tried to test the hypothesis that PDE inhibition by flavonoids is mainly responsible for the inhibition of platelet function. The most potent flavonoid for PDE inhibition is amentoflavone, a dimeric biflavonoid, which inhibits beef heart PDE with an IC_{50} of 0.1 μM /34/. However, amentoflavone does not inhibit the aggregation of intact human platelets at concentrations up to 200 μM. The semisynthetic derivative amentoflavone hexaacetate (AmAc) was synthesized /35/. AmAc has the following properties : a) it inhibits human platelet aggregation with an IC_{50} of 1 μM against ADP- and 3 μM against collagen-induced aggregation ; b) it inhibits cAMP PDE from human platelets with an IC_{50} of 7 μM; c) it increases total platelet cAMP levels in the presence of PGE_1.

A very potent compound on an isolated enzyme system in vitro (PDE) is not necessarily active on an intact cell. Thus inhibition of platelet aggregation by potential drugs remains an efficient and simple pharmacological screening method.

CONCLUSION

Flavonoids may be an important class of anti-platelet compounds of potential therapeutic value in man. However, some drawbacks or difficulties remain : a) they are weakly active in plasma in vitro; b) very high doses may be required to obtain an inhibitory effect;

c) bioavailability is not always optimal; d) the absence of carcinogenic activity is still a matter of debate.

The main advantages of flavonoids as anti-platelet agents are : a) a rise in cAMP secondary to PDE inhibition which inhibits almost any type of platelet activation; b) other biochemical properties, such as cyclooxygenase inhibition, which contribute to the effect on platelet function; c) their low toxicity; d) the wide variety of chemical structures available.

Thrombosis and atherosclerosis are the result of complex pathological interactions involving platelets and leukocytes, the coagulation system, the components of the vessel wall and hemodynamic parameters. Flavonoids may represent an interesting new therapeutic approach because, in addition to their anti-platelet properties, they can interfere with other steps implicated in the development of vascular diseases. Thus, flavonoids inhibit prostaglandin metabolism and the synthesis of mediators of inflammation /30/, alter polymorphonuclear leukocyte response to stimulation /36/, reduce vascular permeability /37,38/, protect endothelial cells /4,5,6/ and modify blood rheological parameters /39/.

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SOME EFFECTS OF FLAVONOIDS ON MAMMALIAN CELL SYSTEMS

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A remarkable literature exists regarding the plant flavonoids, especially their occurrence and distribution in the plant kingdom, and their chemistry and roles in plant physiology (1). The role of flavonoids in mammalian physiology through dietary intake, and their possible importance as disease-alleviating agents or, indeed, as disease-producing agents, is still regrettably enigmatic. Numerous investigations clearly suggest that under particular experimental conditions certain flavonoids, depending on structure, possess antiinflammatory, antiallergic, and antiviral activity (2-4), while others clearly indicate that some flavonoids, again depending on structure, possess mutagenic and potentially carcinogenic activities (5). At first glance, then, one is faced with an apparent paradox, or Yin-Yang situation. Maybe the flavonoids can be both "good" and "bad" environmental (dietary) chemicals. Some aspects of flavonoid biology with respect to their antiallergic, antiinflammatory, and antiviral properties will be briefly reviewed here.

Several early reports suggested that certain flavonoids possessed antiallergic and antianaphylactic activity (4). Solidly planned clinical studies have not been performed, however. Early workers considered that the beneficial effects of flavonoids might be attributed to their vitamin C and epinephrine-sparing activity. Indeed, the vitamin C sparing activity can be attributed to antioxidant properties of certain flavonoids (6) and later it was shown that some flavonoids inhibit catechol-O-methyltransferase thus explaining their epinephrine-sparing activity (7). A mechanistic basis for antiallergic activity was first suggested by the in vitro experiments of Fewtrell and Gomperts (8,9), and Bennett et al (10), who showed that certain flavonoids were capable of inhibiting histamine release from rat peritoneal mast cells stimulated by secretogogues such as antigen, concanavalin A, and chemoattractant peptides. We followed these important observations with a series of experiments utilizing the human basophil histamine release system, an in vitro model of IgE-dependent immediate hypersensitivity reactions, that might portend clinical value for flavonoids in disease states such as asthma, allergic rhinitis, and urticaria in which mast cells and basophils play an important pathogenetic role. It should be noted that naturally occurring flavonoids are structurally related to the antiallergic drug cromolyn.

Initially we studied the common flavonoid, quercetin, for its effect on antigen-induced histamine release from basophils of subjects with ragweed allergy (11). Quercetin caused a concentration-dependent inhibition of histamine release (5-50 μM) and it was more effective at low antigen concentrations, instantaneous in onset of action in antigen-stimulated cells, inhibitory to the histamine release-augmenting effect of D_2O, not significantly affected by increased buffer Ca^{2+} concentrations, and not augmented by theophylline. The latter observation plus the fact that quercetin did not increase leukocyte cyclic-AMP concentrations suggests that the quercetin effect is not cyclic AMP-dependent. Exposure of leukocyte suspension to quercetin (50 μM) for 30 minutes followed by washing and addition of antigen permitted normal histamine release. Only antigen-activated cells, therefore, possess a quercetin-sensitive biochemical system(s) required for basophil granule exocytosis. Subsequently we analyzed the effect of 22 different flavonoids of different chemical classes on antigen-induced basophil histamine release and found some interesting structure-activity relationships (12).

Since antigen-induced basophil histamine release was clearly affected by certain flavonoids but not by others, we decided to study the effects of 11 flavonoids representing five separate chemical classes on the release of histamine from human basophils stimulated by six different secretogogues (antigen, anti-IgE, concanavalin A, ionophore A23187, the chemoattractant peptide, f-MetLeuPhe, and the tumor promoting phorbol ester, tetradecanoyl phorbol acetate (TPA). Unique structure activity relationships were detected (13) showing that the flavonols (3-hydroxy compounds: quercetin and fisetin) and the flavone, apigenin, were the most active inhibitors except that fisetin was relatively inactive against f-MetLeuPhe. Of the three flavones (flavone, apigenin and tangeretin), apigenin was consistently active against each stimulus; flavone and tangeretin, on the other hand, exhibited a wide range of activity being quite active against some stimuli but totally inactive against others. The flavanone, hesperetin, and the flavanonol, taxifolin (dihydroquercetin), showed little or no inhibitory activity against any secretogogue. Thus saturation of the C2-3 bond (with decreased planarity of the heterocyclic ring) is associated with lack of activity regardless of the stimulus to histamine secretion. Similarly, the glycosides, rutin (a 3-O-glycoside) and naringin (a 7-O-glycoside), lacked inhibitory activity possibly because of an inability to cross the cell membrane and enter the cytoplasm, or because the glycosidic groups sterically hinder a portion of the molecule necessary for inhibitory activity. The open chain flavonoid analogs, chalcone and phloretin, were both quite good inhibitors except that phloretin was inactive against antigen. Between 5 µM and 50 µM a considerable spread of inhibitory activity was seen with quercetin, apigenin, fisetin, and phloretin and to a lesser extent with tangeretin, flavone, and chalcone. That is, a strikingly variable sensitivity of different stimuli to inhibition by flavonoids of different structures exists. With quercetin, fisetin, and apigenin, there was an obvious predilection for inhibition of the IgE-dependent ligands, i.e. antigen, anti-IgE, and con A (at all concentrations for quercetin). However, it should be noted that the IgE-dependent ligands, antigen and con A, have been shown to differ in the mechanism by which they stimulate basophil histamine release and in their sensitivity to inhibitory agents (14). The same can probably be said for the other secretogogues (13). It is also of interest that all of the compounds active against receptor-mediated histamine release (especially flavone) are also effective against the non-receptor-dependent secretagogue, ionophore A23187, but whether this "anti-ionophoric" activity represents inhibition of transmembrane Ca^{2+} uptake or inhibition of intracellular Ca^{2+} translocations or some other effect is unknown at present. Indeed, the most striking action of the parent compound, flavone, was inhibition of ionophore-induced histamine release. Of all the flavonoids studied only the open chain congener, chalcone, caused 85-100 percent inhibition of histamine release at the highest concentration examined (50 μM), regardless of the nature of the stimulating agent. Based on the results of these experiments it would appear that various secretogogues utilize or activate biochemical pathways leading to secretion that differ subtly from one stimulus to another, since certain flavonoids active against one or more stimuli may not be effective against others.

The inhibition by several flavonoids of basophil and mast cell histamine release stimulated by various agents caused us to wonder whether naturally occurring flavonoids might also affect the outcome of other cell activation processes such as lymphocyte and neutrophil activation and agonist-induced smooth muscle contraction. It was of considerable interest to find, therefore, that the generation of cytotoxic lymphocytes (CTL) in mixed allogeneic murine spleen cell cultures and the cytotoxic effect of the CTL on a specific target, P815 mastocytoma cells, was inhibited by quercetin, apigenin, fisetin, hesperetin and chalcone, but not by rutin, naringin or catechin (15,16). Thus cellular activation caused by cell-cell interactions is inhibited by certain flavonoids with structure-activity relationships generally similar to those found with the basophil histamine release systems.

The experiments with agonist-induced smooth muscle contraction also showed that certain flavonoids can affect the contractile process (unpublished observations). These studies were performed with guinea pig ileum longitudinal smooth muscle. Quercetin, hesperetin and tangeretin had effects on both the phasic and tonic responses of the muscle to histamine, acetylcholine, and prostaglandin E, but naringin was inactive as it was in the basophil histamine release experiments (12) and antiviral experiments (see below).

Stimulation of neutrophils with opsonized zymosan, for example, is associated with the production of superoxide anion (O_2^-), generation of the chemiluminescence phenomenon, increased glucose metabolism through the hexose monophosphate pathway and release of lysosomal enzymes. We examined the effect of quercetin and several other flavonoids on some of these processes (17) as well as the effect of quercetin on ^{3}H-arachidonic acid release from prelabelled neutrophils stimulated with opsonized zymosan (18). Nine flavonoids (hesperetin, fisetin, apigenin, taxifolin, rutin, tangeretin, cathechin, chalcone, and quercetin) were all quite active in inhibiting the generation of chemiluminescence and quercetin was a moderately effective inhibitor of O_2^- generation and oxygen uptake. Of the nine flavonoids only quercetin and chalcone caused greater than 20 percent inhibition of beta-glucuronidase release; hesperitin and catechin caused a reproducible augmentation of beta-glucuronidase release. Superoxide formation involves activation of NADPH oxidase; it is of interest, therefore, that quercetin has been shown to inhibit NADPH oxidase (19). ^{3}H-Arachidonic acid release from prelabelled neutrophils stimulated with opsonized zymosan was also moderately inhibitied by quercetin (18) suggesting inhibition of the activation of phospholipase A_2 or perhaps also of diacylglycerol lipase, both of which would induce the release of arachidonic acid from the parent phospholipids.

The antiviral activity of certain flavonoids has long been recognized. Hungarian investigators showed that some flavonoids exhibited inhibitory activity against several viruses (cf. 20). Recently a flavonoid was extracted from an ancient Chinese herbal medicine plant and characterized as 4', 5-dihydroxy-3, 3', 7-trimethoxyflavone (21). It exhibited marked inhibitory activity *in vitro* against rhinoviruses and coxsackie viruses in tissue culture and was shown to inhibit intracellular replication possibly by interferring with a process occurring between viral uncoating and initiation of viral RNA synthesis.

We have studied the inhibitory activity of quercetin, catechin, hesperitin, and naringin (representing four different flavonoid chemical classes) against respiratory syncytial virus (RSV), parainfluenza virus type 3 (Pf-3), herpes simplex virus type 1 (HS-1), and polio virus type 1 (PV-1). The results indicated that quercetin possessed both antiinfective and antireplicative activity against each virus; hesperetin had no antiinfective activity but possessed antireplicative effects against each virus; catechin showed antiinfective properties against RSV and HSV-1 only and lacked antireplicative activity altogether; and naringin totally lacked antiinfective and antireplicative activity (20). Some synthetic flavonoids, e.g. dichloroflavan, possess powerful antirhinovirus activity (22) but dichloroflavan lacked in vivo activity in man (23).The possible role of dietary flavonoids as antiviral agents has not been determined.

The above briefly reviewed evidence indicates that certain flavonoids affect the responses of various cells to activating stimuli of one kind or another: basophil histamine release in response to six different secretogogues; neutrophils in response to opsonized-zymosan (anaphylatoxin); lymphocytes in response to mitogenic stimulation (concanavalin A) and allogeneic cell-cell interaction leading to generation of CTL and the effector function of the CTL on another cell target; smooth muscle in response to contractile agonists; and viral infection and replication. What do all of these cell activation possesses have in common? One likely possibility is activation of phospholipase C and initiation of the phosphatidylinositol cycle, a frequent if not universal accompaniment of cell activation in many different cell types (24). Indeed, inhibition of phospholipase C in rabbit neutrophils by quercetin has been demonstrated (25). Another possibility is activation of phospholipase A_2 and the methyltransferases, enzymes shown to be involved in mast cell mediator secretion (26). As noted above, at least in neutrophils, activation of NADPH oxidase is required for O_2^- generation in neutrophils. Undoubtedly other enzyme systems are activated in the process of cell-ligand interactions. Another enzyme system involved in signal transduction across cell membranes is the Ca^{2+}-and phospholipid-dependent protein kinase known as protein kinase C. This enzyme appears to be involved in mast cell mediator secretion (27) as well as in a variety of other cell processes. It is of interest that at least one flavonoid, quercetin, has been shown to inhibit protein kinase C in rodent brain (28). It is well recognized that a number of flavonoids have potent effects as inhibitors of a variety of enzymes. Some of these enzymes are Ca^{2+}-and calmodulin-dependent. One can't help but wonder whether the multiplicity of enzyme inhibitory effects may be related to the ability of flavonoids to interact with calmodulin and inhibit its essential role in the process of enzyme activation. It is of interest that one report indicates that quercetin does indeed interact with calmodulin(29).

While the in vitro data from many experiments are sound, there is still a need for more studies on the in vivo effects of flavonoids. How well are they absorbed from the gastrointestinal tract? In what tissues are they distributed and how long do they stay? How are they metabolized and excreted? Do dietary flavonoids have any effect on allergic, inflammatory or carcinogenic processes? While some data are available in animals (4,30), very few exist for man. Quercetin, so often an active compound in many in vitro systems, is poorly absorbed (if at all) in man (31). More data on pharmacokinetics in man is needed.

In summary, the naturally occurring flavonoids possess many biochemical properties which suggest that they might act as natural biologic response modifiers affecting many cells systems involved in immunity, inflammation and allergic reactions.

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COMPARATIVE EFFECTS OF SOME FLAVONOIDS ON CYCLOOXYGENASE AND LIPOXYGENASE ACTIVITIES IN DIFFERENT CELL SYSTEMS OR SUBFRACTIONS

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INTRODUCTION

In recent years, flavonoids have been used as antiinflammatory and antiasthmatic agents. These activities may be related to an inhibitory effect on the biosynthesis of icosanoids involved in the physiopathology of the inflammatory reaction. It has been claimed that flavonoids inhibit various enzymes involved in the arachidonic acid cascade such as phospholipase A_2 (1) cyclooxygenase (2, 3) lipoxygenases (2, 3, 4, 5, 6, 7) and 9-hydroxyprostaglandin dehydrogenase (4). In this study, we examined the effects of thirty flavonoids (free or glycosylated) on cyclooxygenase in two cellular systems : bull seminal vesicle microsomes and human platelets, and on 12-lipoxygenase from human platelets.

MATERIALS AND METHODS

1°) Reagents

[1-^{14}C]-arachidonic acid (58 mCi/mmol) was purchased from the Radiochemical Center (Amersham, England). Unlabeled arachidonic acid was from Sigma Chemical Company (Saint Louis, Mo., USA) and was stocked at -20 °C in an ethanol solution ; glutathion was from Fluka (Switzerland) and hydroquinone from Prolabo (France). Buffer substances, all analytical grade, were from Merck (USA). Flavonoids were kindly supplied by P. Fabre (Castres France), and by Sigma (St Louis, USA), and Roth (Karlsruhe, FRG.).

2°) BSV Cyclooxygenase

a) Microsome preparation : All manipulations were carried out at 4 °C. Frozen bull seminal vesicles were thawed, homogenized in a Potter tube with two volumes of 0.1 M phosphate buffer, pH 7.2, containing 1 mM EDTA and 1 mM cystein. The homogenate was centrifuged at 20,000 x g, for 20 min, in an L 3-50 Beckman centrifuge. The supernatant was centrifuged at 100,000 x g, for 3 h, and the pellet was resuspended in 0.1 M Tris-HCl buffer, pH 8, containing 1 mM EDTA, and resedimented after centrifugation at 100,000 x g., for 3 h. The washed microsomal fraction was resuspended in this buffer to yield 25 to 30 mg protein/ml and divided into small fractions which were stored at -20 °C. Protein concentrations were measured using bovine serum albumin as standard.

b) Prostaglandin synthetase assay : Each incubation medium contained bull seminal vesicle microsomes (2.5 to 5 mg protein), [1-^{14}C]-arachidonic acid (0.2 μCi/10 μg), reduced glutathion (1 mM), hydroquinone (0.2 mM), human oxyhemoglobin (0.3 μM) and inhibitor (10^{-5}-10^{-8} M) in 4 ml of 50 mM Tris-HCl buffer, pH 8. The mixture was incubated, for 20 min at 37°C. The reaction was stopped by the addition of 1 M citric acid. Samples were extracted twice with 5 ml ethyl acetate. The organic phase was washed with water and evaporated to dryness under reduced pressure at 40 °C. The prostaglandins were separated from the arachidonic acid by thin-layer chromatography on silica gel G using benzene/dioxane/acetic acid 20:10:1. The plate was scanned with a Berthold radiochromatogram scanner. Areas corresponding to the radioactive peaks (arachidonic acid and PGE_2) were scraped and the radioactivity was counted in a Packard Tricarb liquid scintillation spectrometer.

3°) Platelet cyclooxygenase and 12-lipoxygenase

a) Platelet preparation : Blood was collected by venipuncture from volunteers who had not taken any drug for three weeks. Six volumes of blood were collected in 1 volume of ACD (citric acid-citrate-dextrose, 66 mM, 85 mM, 115 mM). Platelet-rich plasma (PRP) was obtained by centrifugation (300 x g, 8 min) at room temperature. 1 volume EDTA (10 mM) was added to 10 volumes of PRP. The PRP was then centrifuged (900 x g, 10 min), and the platelet pellet was washed twice with Tris-HCl buffer (15 mM, pH 7,4 containing 134 mM NaCl, 1 mM EDTA, 5 mM glucose).

b) Cyclooxygenase and 12-lipoxygenase assays : 500 µl of washed human platelets (10^8 platelets/ml) was preincubated with flavonoids for five minutes at 37 °C. [1-^{14}C]-arachidonic acid (180 000 dpm) was then added. After ten minutes, incubates were acidified to pH 4 with 2 M citric acid, and arachidonic acid and its metabolites were extracted twice with 3 volumes of diethylether. The organic phase was taken to dryness under a nitrogen stream. The dry residue was redissolved in 100 µl ethylacetate, quantitatively applied onto silica gel TLC plates (Merck) and developed in diethylether/petroleum ether/acetic acid (50:50:1, v/v). Radioactive compounds were located and quantified using a Numelec TLC scanner.

Figure 1 shows a typical radiochromatogram obtained with washed human platelets without flavonoids. Three major metabolites were formed : TxB_2 and HHT via the cyclooxygenase pathway, and 12 HETE via the 12 lipoxygenase pathway. Almost all the arachidonic acid was converted to metabolites.

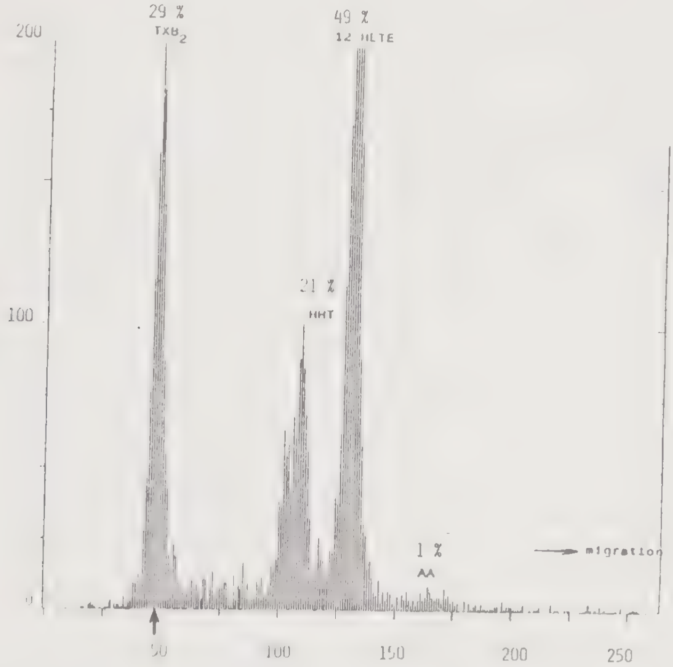


Fig. 1 : CO and 12 LO activities in platelets :
sample radiochromatogram obtained in control.

RESULTS

Flavonoids were tested at 0.1 mM. When inhibition was higher than forty percent at this concentration, we determined the inhibitor concentration resulting in fifty percent inhibition, IC_{50} (Figures 2, 3a, 3b). In the platelet model, some flavonoids, such as fisetin, inhibited both CO and 12 LO (Figure 4) ; others, such as flavanone, inhibited only CO, and 12 HETE formation was apparently stimulated (Figure 5). This stimulation may have been due to either an activation of 12-LO, or a higher availability of the substrate for 12 LO, since CO was inhibited. The second hypothesis seems to be the most probable since indomethacin, which inhibited CO but had no effect on 12 LO, produced similar "apparent" LO activation : the more CO was inhibited, the more LO was apparently activated.

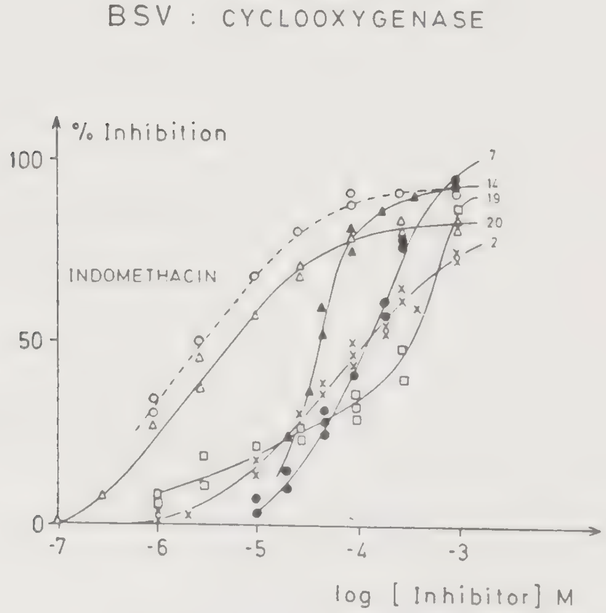
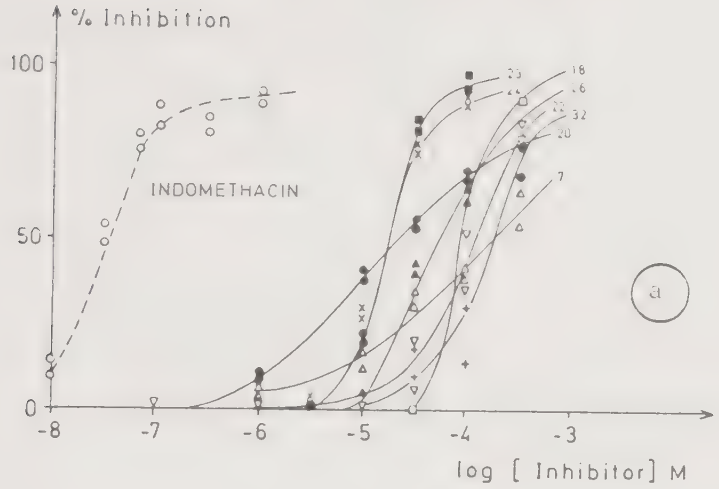


Fig. 2 : Effect of various concentrations of different flavonoids on BSV CO activity.

PLATELETS : CYCLOOXYGENASE



PLATELETS : 12-LIPOXYGENASE

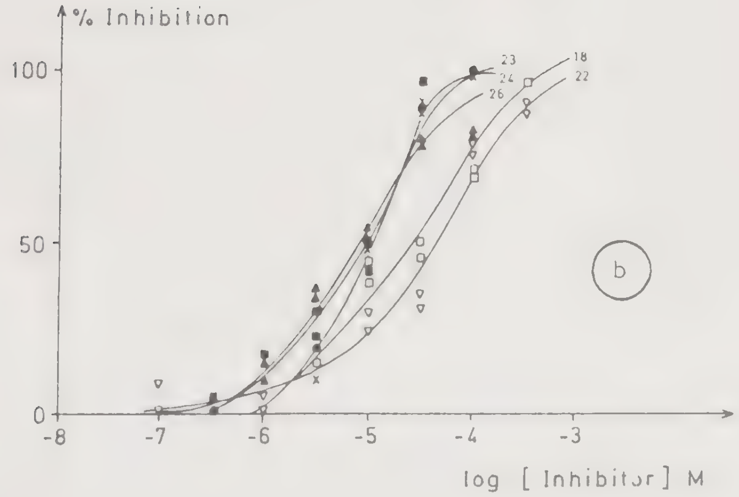


Fig. 3 : Effect of various concentrations of different flavonoids on : a) platelet CO
b) platelet 12-LO

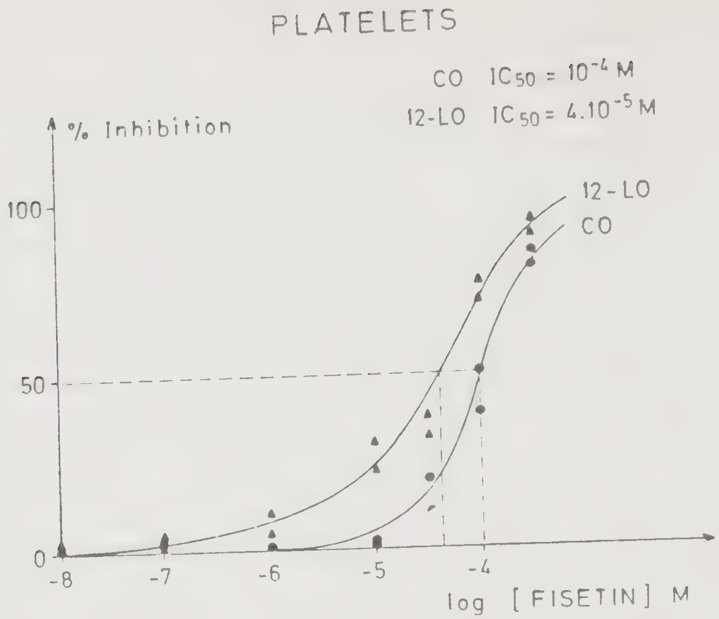


Fig. 4 : Effect of various concentrations of fisetin on platelet CO and 12 LO.

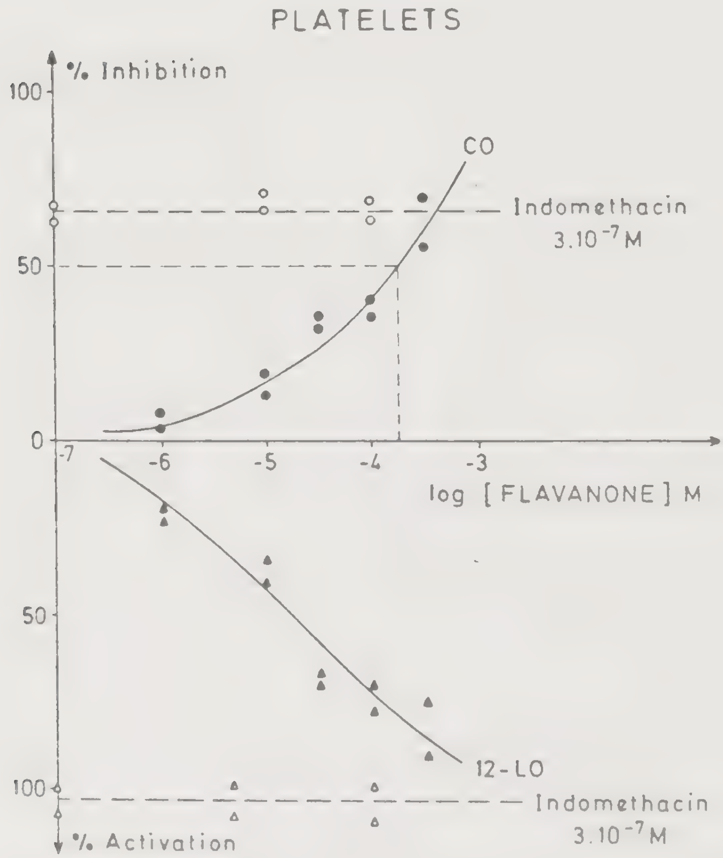


Fig. 5 : Effect of various concentrations of flavanone on platelet CO and 12 LO.

Results are presented in Tables 1 - 5.

Table 1 : flavan and some derivatives

Flavan (1) is a weak inhibitor of BSV cyclooxygenase. In the platelet model, neither CO nor LO were inhibited by flavan . Catechin (2) inhibited BSV cyclooxygenase, and the stereochemistry at carbons 2 and 3 had no influence on the inhibitory effect. Catechin had little inhibitory effect on platelet CO, and epicatechin had no effect. None of these compounds inhibited 12 LO.

Table 2 : flavanone and some derivatives.

Cyclooxygenase inhibition

Flavanone (7) differs from flavane (1) with respect to its carbonyl at position four. It was a better inhibitor than flavan in the two models.

Hydroxyl groups at positions 5, 7 and 4' suppressed the inhibitory effect (naringenin, 8). This loss of activity was probably due to the 4'-hydroxyl group because hesperetin (9) had the same activity as flavanone (7).

Taxifolin (13) had the same inhibitory effect on platelets as catechin (2).

The O-glycosylation at R_1 was not favorable : compare hesperidin (10) with hesperetin (9) ; an inactive compound, naringenin (8), was still inactive after glycosylation (naringin 11).

12 LO inhibition

Only fustin (12) and taxifolin (13) had slight inhibitory effects. Note that these compounds have two hydroxyl groups at 3' and 4'.

Table 3 : flavone and some derivatives.

CO inhibition

Flavone (14) differs from flavanone (7) with respect to its conjugated double bond at position two. It was a better inhibitor of BSV CO, but flavone and flavanone had the same inhibitory effect on platelet/CO.

The presence of two hydroxyl groups at positions 5 and 7 decreased the inhibitory effect (15) and another hydroxyl group at 4' yielded apigenin (16) which was totally inactive.

The same results were obtained with naringenin (8) and flavanone (7).

Table 1 : Flavan and some derivatives

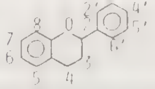
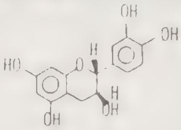
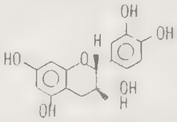
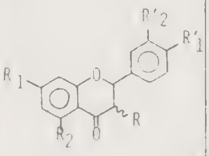
FORMULA	N°	NAME	C O		12 L O
			BSV	PLATELETS	PLATELETS
			% I AT 0.1MM (IC_{50} µM)		% I AT 0.1 MM
	1	FLAVAN	20	0	0
	2	(+) CATECHIN	45 (130)	20	0
	3	(-) CATECHIN	50	20	0
	4	(±) CATECHIN	50		
	5	(-) EPICATECHIN	55	0	0
	6	(±) EPICATECHIN	50		

Table 2 : Flavanone and some derivatives

FORMULA	N°	NAME	R	R_1	R_2	R'_1	R'_2	C O		12 L O
								BSV	PLATELETS	PLATELETS
								% I AT 0.1 MM		(IC_{50} µM)
	7	FLAVANONE	H	H	H	H	H	40 (160)	45 (200)	0
	8	NARINGENIN	H	OH	OH	OH	H	0	0	0
	9	HESPERETIN	H	OH	OH	OCH_3	OH	50 (100)	15	0
	10	HESPERIDIN	H	GRO	OH	OCH_3	OH	0	0	0
	11	NARINGIN	H	GRO	OH	OH	H	0	0	0
	12	FUSTIN (DIHYDRO-FISETIN)	OH	OH	H	OH	OH	15	10	20
	13	TAXIFOLIN (DIHYDRO-QUERCETIN)	OH	OH	OH	OH	OH	0	20	30

GRO = GLUCOSE-RHAMNOSE-O

Table 3 : Flavone and some derivatives

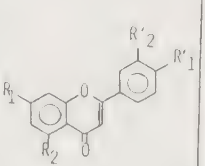
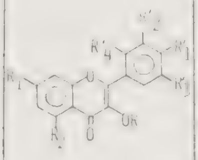
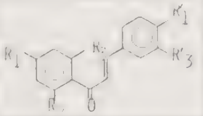
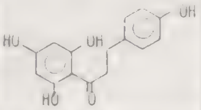
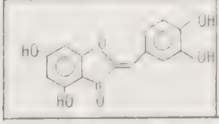
FORMULA	N°	NAME	R_1	R_2	R'_1	R'_2	C O		12 L O
							BSV	PLATELETS	PLATELETS
							% I AT 0.1 MM		(IC_{50} µM)
	14	FLAVONE	H	H	H	H	80 (50)	30	0
	15	CHRYSIN	OH	OH	H	H	30	20	10
	16	APIGENIN	OH	OH	OH	H	0	0	15
	17	ACACETIN	OH	OH	OCH_3	H	10	10	0
	18	LUTEOLIN	OH	OH	OH	OH	30	75 (60)	70 (40)

Table 4 : Flavonol and some derivatives

FORMULA	N°	NAME	R_1	R_2	R'_1	R'_2	R'_3	R'_4	3-OH	CO BSV	CO PLATELETS	12 LO PLATELETS
										% I AT 0.1MM (IC50 uM)		
	19	FLAVONOL	H	H	H	H	H	H	H	[illegible]	[illegible]	
	20	GALANGIN	OH	OH	H	H	H	H	H	[illegible]	[illegible]	0
	21	KAEMPFEROL	OH	OH	OH	H	H	H	H	0	50	0
	22	FISETIN	OH	H	OH	OH	H	H	H	20	[illegible] (75)	30 ([illegible])
	23	QUERCETIN	OH	OH	OH	OH	H	H	H	0	[illegible] (15)	100 (10)
	24	RHAMNETIN	OCH_3	OH	OH	OH	H	H	H	[illegible]	[illegible] (14)	100 (11)
	25	MORIN	OH	OH	OH	H	H	OH	H	0	10	0
	26	MYRICETIN	OH	OH	OH	OH	OH	H	H	0	[illegible] (40)	[illegible] (7)
	27	QUERCITRIN	OH	OH	OH	OH	H	H	RO	0	5	40
	28	RUTIN	OH	OH	OH	OH	H	H	GRO	0	0	0

RO = Rhamnose-O
GRO = Glucose-Rhamnose-O

Table 5 : Aurones and chalcones

FORMULA	N°	NAME	R_1	R_2	R_3	R'_1	R'_3	CO BSV	CO PLATELETS	12 LO PLATELETS
								% I AT 0.1 MM (IC_{50} uM)		
	29	CHALCONE	H	H	H	H	H	80 (50)	5	0
	30	HESPERIDIN METHYL CHALCONE	GRO	OH	OCH_3	OCH_3	OCH_3	0	0	0
	31	PHLORETIN						60 (45)	0	40
	32	AUREUSIDIN						20	20 (170)	65 (55)

GRO = Glucose-Rhamnose-O

The suppression of the inhibitory effect was probably due to the electroattracting phenolic group at 4' ; its methylation (acacetin 17) or its incorporation in a o-diphenolic structure (luteolin 18) restored its inhibitory effect.

12 LO inhibition

The strongest inhibitory effect was observed with luteolin (18), which, like fustin (12) and taxifolin (13), has two hydroxyl groups at 4' and 5'.

Table 4 : Flavonol and some derivatives

CO inhibition

Flavonol (19) was less active than flavone (14). Its activity was similar to that of flavanone (7). Two hydroxyl groups at 5 and 7 yielded galangin (20) which was the best inhibitor of BSV (IC_{50} = 5.5 μM, similar to that of indomethacin, IC_{50} = 3 μM). Galangin inhibited platelet CO (IC_{50} = 20 μM).

The addition of a hydroxyl group at 4' yielded kaempferol (21), which was totally inactive.

The introduction of more hydroxylated groups yielded compounds that had no effect on BSV cyclooxygenase but inhibited platelet cyclooxygenase and 12 LO.

12 LO inhibition

As in the other series, the presence of two hydroxyl groups at positions 4' and 5' was a prerequisite for LO inhibition : fisetin (22), quercetin (23), rhamnetin (24) and myricetin (26) were inhibitors, kaempferol (21) and morin (25) were not inhibitors.

O-glycosylation was not favorable : quercitrin (27) and rutin (28) were less active than quercetin (23).

Table 5 : Aurones and chalcones

Chalcone (29) and phloretin (31) had the same effect as flavone (14) on BSV CO.

Aureusidin (32) and luteolin (18), which are sterically identical, had the same effect on BSV CO (at 0.1 mM, 20 % inhibition and 30 % inhibition respectively) and on platelet 12 LO (IC_{50} = 55 μM and 40 μM respectively) ; platelet CO was less inhibited by aureusidin than by luteolin (IC_{50} = 170 μM and 60 μM respectively). It should be noted that aureusidin has the two hydroxyl groups at positions 4' and 5', which are prerequisites for a good inhibition of 12 LO.

DISCUSSION

In this study, a discordance was observed when flavonoids were tested for their inhibitory effect on two different models of CO : BSV and platelets. Among the flavonol derivatives, fisetin (22), quercetin (23), rhamnetin (24), myricetin (26) had an inhibitory effect only on platelet CO and no effect on BSV CO. Discordances also could be noted between our results and those in the literature. Baumann et al. (2) reported that galangin and morin had the same inhibitory effect on rat renal medulla CO (IC_{50} = 120 µM), whereas we found an IC_{50} = 5.5 µM for galangin, and morin had no inhibitory effect on BSV CO. However, like these authors, we found the same IC_{50} for catechin and epicatechin (IC_{50} = 130 µM). Sekiya reported that quercetin, rutin, hesperetin, hesperidin, chrysin, and naringenin were weak inhibitors of platelet CO and 12 LO (5). Our results agree with respect to rutin, hesperedin, chrysin and naringenin, but we found quercetin to be active against platelet CO and 12 LO (IC_{50} = 15 µM and 10 µM respectively) and hesperetin was active against BSV CO (IC_{50} = 100 µM). These divergences may be due to the origin of the microsomal preparations, as other authors have suggested (8). Furthermore, Wauwe and Goosens (9), using the same model as ours (intact human platelets) in a study of the effects of five antioxidative agents (including quercetin) on CO and 12-LO activities, found the same IC_{50} values as ours with respect to quercetin s effect on CO (IC_{50} = 19 µM) and 12-LO (IC_{50} = 6 µM). The best model for therapeutical applications seems to be platelet CO.

This study shows that at least two flavonoid series can be distinguished : those which inhibit only CO (flavanone, hesperetin, flavone, and galangin, the latter being the best inhibitor) and those which inhibit both CO and 12 LO (fisetin, quercetin, rhamnetin and myricetin). The results concerning 12 LO are in agreement with those of Landolfi et al. (10), who showed that out of 18 flavonoids, only two flavonols, quercetin (with a catechol ring) and myricetin (with a pyrogallol ring), inhibit platelet LO. Kaempferol and morin (flavonols with phenol and resorcinol rings) are much less potent inhibitors of LO, whereas flavones and flavanones are inactive. Gryglewski et al. (3) showed that quercetin is the most active inhibitor of soybean 15 LO (IC_{50} = 13 µM), horse platelet 12 LO (IC_{50} = 1.3 µM) and human platelet 12 LO (IC_{50} = 4.4 µM). These authors question whether flavonoids with phenol, catechol or pyrogallol rings could be lipoxygenase inhibitors, simply because of the free radical scavenging properties of these moieties. This structural condition is certainly insufficient, since the lipoxygenase-inhibiting properties of catecholic and pyrogallic flavonoids are drastically reduced or abolished if the 3-hydroxyl group in the benzopyrone ring of quercetin is blocked with a sugar (quercitrin, rutin) or if flavone is replaced by a flavanone skeleton (fustin, taxifolin).

Gryglewski et al. (3) showed that quercetin has a dual effect on CO activity in ram seminal vesicle microsomes. Quercetin stimulates CO when a high concentration of substrate (100 μM) is used. At low concentrations of arachidonic acid (1.6 μM), quercetin inhibits the enzyme. These authors give a possible explanation of this fact, based also on the experiments of Kulmacz and Lands (11). Lipid peroxides (PGG_2, HPETE) in a concentration range of 10^{-9}-10^{-7}M initiate oxygenation of arachidonic acid, but, when lipid peroxides accumulate at concentrations higher than 10^{-6} M, CO inhibition occurs. Lipid peroxides and free radicals can activate or inhibit CO in this way, depending on the amount of the substrate available for their generation. According to Gryglewski (3) quercetin behaves like a typical free radical scavenger : at high substrate concentrations, quercetin removes the surplus of inhibitory free radicals and then stimulates R SVM CO, whereas at low substrate concentrations, it scavenges small amounts of activating free radicals and thus inhibits the enzyme. Baumann et al. (12) compared the radical scavenging properties of 45 flavonoids and related substances, and they concluded that a direct involvement of $O_2^{-\bullet}$-radicals seems to be improbable.

Egan (13) and Dewhirst (14) have supposed that the critical element for the inhibitory effect on CO activity is the ability of the compound to complex a metal ion that might play an important role in enzyme function. Heme, for instance, is required for both the dioxygenase and peroxidase activity of CO (11). 12 LO is an enzyme with a ferric or ferrous ion, and is inhibited by usual complexants (15). Using spectrophotometric and polarographic analysis, Clark and Geissman (16) studied the chelation of several flavonoids with metals. They concluded that the important complex-forming elements were the catechol structure and the 3-hydroxy 4-keto groups ; the presence of the 2-3 double bond in the flavones stabilizes the metal complex by contributing to the resonant hybrid forms. Our results are in total agreement with this concept. We clearly show the importance of the 3' and 4' hydroxyl groups in the inhibitory effect on 12 LO, whereas flavanone derivatives (taxifolin and fustin), which have these hydroxyl groups but no 2-3 double bond, are weak inhibitors. We postulated that flavonoids could act as specific chelators, i.e. compounds with a chelator group included in a structure that a recognizable by the enzyme, thus explaining the difference in activity toward CO and LOs.

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OXIDATION OF SUITABLE DRUGS BY HORSERADISH PEROXIDASE/HYDROGEN PEROXIDE, RESULTS IN THE OXYGENATION OF ARACHIDONIC ACID TO FORM HYDROPEROXYEICOSATETRAENOIC ACIDS IN A REVERSE COOXIDATION PROCESS

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INTRODUCTION:

Arachidonic acid (AA, 20 : 4) and related polyunsaturated fatty acids are metabolized either by prostaglandin endoperoxide synthase (PES) to produce the prostaglandins (PGs), prostacyclin or thromboxane, or by lipoxygenases to generate hydroperoxy derivatives (HPETEs). Although cyclo-oxygenation is the initial reaction catalyzed by PES, the purified enzyme also exhibits a peroxidase-like activity which generates the hydroxyendoperoxide PG H with the liberation of an oxidizing species.

Lipoxygenases oxygenate AA to produce HPETEs which in mammalian systems may be further metabolized either to leukotrienes or to hydroxy derivatives by peroxidatic attack. Besides the physiological synthesis of cyclooxygenase products from AA the peroxidase component of PES catalyzes the oxidation of a wide variety of xenobiotics. This type of drug activation which is not mediated by the cytochrome-P_{450}-system was described by MARNETT et al. /1/ and was called cooxidation. Especially polycyclic hydrocarbons and aromatic amines are cooxygenated during the reduction of PG G to PG H by the peroxidase activity of PES. In contrast to cytochrome-P_{450}-metabolism, these cooxidation products appear to be formed by radical or electron transfer mechanisms. Essential peroxidase cosubstrates of this cooxidation process are PG G, which is generated during the cyclooxygenation of AA, hydrogen peroxide or other hydroperoxides. Thus, the cooxidation of xenobiotics to reactive radical products only requires a highly active peroxidase and suitable hydroperoxides. The resulting radical intermediates avidly react with proteins, glutathione, fatty acids or DNA. On the other hand, similar radical intermediates of xenobiotics may be generated by non-enzymic physicochemical attack: recently, BÖSTERLING and TRUDELL /2/ reported the formation of halothane radicals by UV-irradiation in vitro and their subsequent interaction with AA to form 5- and 15-HPETEs. Besides PES, another peroxidase - horseradish peroxidase - (HRP, EC 1.11.17) is well known to catalyze the hydroperoxide-dependent oxidation of a wide variety of organic drug substrates (RH) in the presence of hydrogen peroxide or other suitable hydroperoxides /3/.

The aim of our study was to elucidate whether AA may be converted to the biologically important HPETEs in a non-lipoxygenase oxidation system during the cooxidative activation of several xenobiotics (e.g. flavonoids, phenol and benzene derivatives) by HRP in the presence of hydrogen peroxide.

Since several flavonoids are well known to inhibit cyclo-oxygenases and lipoxygenases of different origin /4-9/ we also investigated whether flavonoid or non-flavonoid inhibitors of the AA peroxidation pathways are metabolized by HRP/H_2O_2 to form activated reaction products which may initiate the oxygenation of AA to yield HPETEs.

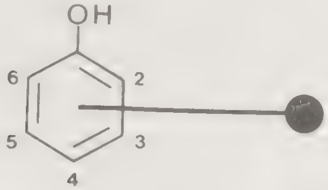
METHODS:

Oxygenation of (1-^{14}C)AA (sp. act. 50 Ci/Mole) was monitored in a mixture containing purified HRP (1-10µg), hydrogen peroxide (0-1000 µM), (1-^{14}C)AA (38 nCi, 2.5-100 µM) and potassium phosphate (50 mM, pH 8.0) in the presence or absence of various HRP-substrates (e.g. flavones, flavonols, ß-O-hydroxyethylflavonoids, phenol and benzene derivatives; 0.1 - 1 mM). (1-^{14}C)AA and its oxygenation products were separated 1) by TLC (ether : petroleum benzine : acetic acid, 50 : 50 : 1) or 2) by selective solvent extraction of non-converted AA according to a procedure developed by KNIPPEL et al. /10/ in our laboratory and were quantitated by LSC. Authentic radiolabelled lipoxygenase products were provided by Amersham, U.K., or by New England Nuclear, F.R.G. Blanks including HRP/H_2O_2 and buffer instead of RH yielded only small amounts (2-5% conversion) of HPETE which is in accordance with GARNER /11/.

RESULTS:

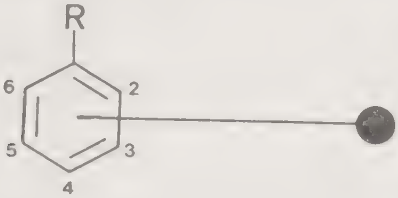
Our studies revealed that many activated radical oxidation products derived from a variety of substrates (see Table 1) can initiate the oxygenation of arachidonic acid (AA) whereby large amounts of hydroperoxyeicosatetraenoic acids (40-50% conversion of AA) were formed, more than 90% of which cochromatographed with 15-HPETE and 15-HETE. Dependent on the chemical reactivity of the primary oxidation products (R*) more or less AA was indi-

Table 1: Activation of phenol and benzene derivatives by HRP/H_2O_2 results in the cooxidation of AA yielding 15-HPETE via a non-lipoxygenase type reaction.



		20 : 4 - COOXIDATION
Phenol	H	-
Catechol*	2-OH	↓
Hydroquinone	4-OH	-
Pyrogallol	2-OH, 3-OH	(+)
o-	2-	++++
m- Cresol	3-CH_3	++++
p-	4-	++++
p-Ethylphenol	4-C_2H_5	++++
p-Hydroxybenzoic acid	4-COOH	(+)
o-	2-	+
m- Acetamidophenol	3-$NHCOCH_3$	++
p- (Paracetamol)	4-	+
p-Nitrophenol	4-NO_2	+
o- Hydroxydiphenyl	2-	++++
p-	4- C_6H_5	++++
Guaiacol	2-OCH_3	++
Adrenalin*	2-OH, 4-CHOH-CH_2NHCH_3	↓
Diflunisal	2-COOH-4-$C_6H_3F_2$-(2,4)	-

* = Peroxidase inhibitor



		R	20 : 4 - COOXIDATION
o- p- Methoxybenzoic acid	2- 4- COOH	OCH_3	+++
Acetylsalicylic acid	2-COOH	$OCOCH_3$	-
5-Aminosalicylic acid	5-COOH	NH_2	-
o- p- Phenylenediamine	2- 4- NH_2	NH_2	-
o-	2-		+
m- Phenetidine	3- NH_2	OC_2H_5	+++
p-	4-		+

rectly cooxidized to HPETEs by the activated products of the HRP/hydrogen peroxide system in a free radical chain reaction cascade whereby the fatty acid oxygenation was initiated by the formation of a carbon-centered free radical. The final reaction of this AA free radical (AA*) with triplet oxygen ($^3O_2$) is the normal biochemical trapping reaction and mimics a classic 15-lipoxygenase-type of reaction. In addition to being oxidized by peroxidases, the substrates (RH) were also cofactors for the final reduction of the resulting HPETEs to the corresponding HETEs.

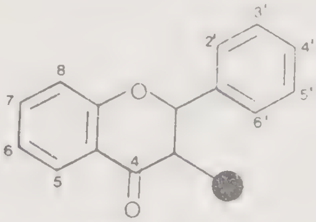
There were several inhibitors of the cyclo-oxygenase and lipoxygenase pathways among the suitable substrates (RH) which may initiate the conversion of AA to 15-HPETE during their HRP-dependent oxidation (Table 2).

Table 2: Activation of cyclo-oxygenase and lipoxygenase inhibitors by HRP/H_2O_2 results in the cooxidation of AA.

PG-SYNTHASE-INHIBITORS	20 : 4 - COOXIDATION
Phenylbutazone	+++
3-Methyl-1-phenyl-2-pyrazolin-5-one	+++
Carprofen	++++
Tiaprofenic acid	++
Mefenemic acid	++++
Benoxaprofen	+++
Paracetamol	++
Piroxicam	+
Naproxen	(+)
Aminopyrine	(+)
Acetylsalicylic acid	-
Diflunisal	-
Indometacin	-
(+)-Catechin	-
Flavonol	+++
LIPOXYGENASE-INHIBITORS	
Phenidone	+++
NDGA	-
Acetonylacetone bisphenylhydrazone	++(+)
Quercetin	-

Among the flavonoids investigated (see Tables 3, 4) only the activation of flavonol and of ß-O-pentahydroxyethylquercetin resulted in a drastic conversion of AA to form 15-HETE.

Table 3: Activation of flavonol by HRP/H_2O_2 results in the cooxidation of AA (20 : 4) yielding 15-HPETE. Flavones and flavonol derivatives were inactive.



FLAVONES ——● : H

7—— }
6,7—— }
7,8—— } -OH
3',4'*—— }
5,7 (Chrysin)—— }

4'-COOH

5,7——OH
5,7——OCH_3

FLAVONOLS ——● : OH

Flavonol (20 : 4 - cooxidation: +++)
Flavonol-methylether
5,7 (Galangin)—— } -OH
5,7,3',4'* (Quercetin)—— }

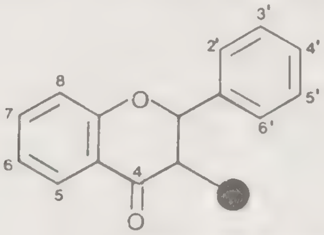
4'-COOH
5,7-OH
5,7-OCH_3

Catechin

----

* = Peroxidase inhibitor

Table 4: Activation of β-O-pentahydroxyethylquercetin by HRP/H_2O_2 results in the cooxidation of AA (20 : 4) yielding 15-HPETE. Other hydroxyethylflavonoids were inactive.



β-O-HYDROXYETHYLFLAVONOIDS

RUTIN ——● : O-Rutinose

4' ——
3',4',7 —— } $-OCH_2CH_2OH$
3',4',5,7 ——

QUERCETIN ——● : OH

4' ——
3',4',7 —— } $-OCH_2CH_2OH$
3',4',5,7 ——

QUERCETIN ——● : OCH_2CH_2OH

3',4',7 ——
(20 : 4 cooxidation: +++) 3',4',5,7 —— } $-OCH_2CH_2OH$

CONCLUSIONS:

Our results confirm the data of Bösterling & Trudell /2/ who described the non-enzymatic formation of 5- and 15-HPETEs from AA by halothane free radicals generated by high power UV-irradiation in vitro. In contrast, our studies reveal that the oxidative metabolism of a wide variety of chemically different compounds, including flavonol, β-O-pentahydroxyethylquercetin and other therapeutically and toxicologically important drugs and xenobiotics, mediated by peroxidases and hydroperoxide cosubstrates triggers the formation of HPETEs and HETEs. Thus, besides being generated by lipoxygenases, these intermediates in the enzymatic formation of leukotrienes may be formed indirectly in the course of mammalian peroxidase reactions when suitable peroxidase substrates (RH) are oxidized.

Scheme 1: Hypothetical mechanisms for the peroxidase-catalyzed activation of xenobiotics and the subsequent formation of HPETE in a reverse cooxidation process

HRP + H_2O_2 —— Compound I + H_2O
RH + Compound I —— Compound II + R*
RH + Compound II —— HRP + R* + H_2O
R* + AA —— AA* + H**R
AA* + $^3O_2$ —— AA-OO*
AA-OO* + H* —— AA-OOH (HPETE)
AA-OOH + 2 RH $\xrightarrow{HRP}$ AA-OH (HETE) + H_2O + 2 R*

Suitable substrates RH are indicated in Tables 1 to 4.

ACKNOWLEDGEMENT:

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FLAVONOIDS INHIBIT ENZYMIC THYROID HORMONE DEIODINATION

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INTRODUCTION

Thyroid hormones control growth, differentiation and basal metabolic rate of human beings and higher animals. The main secretory product of the thyroid gland, the (pro-)hormone L-thyroxine (3,3',5,5'-tetraiodo-L-thyronine, T4) is enzymatically monodeiodinated by iodothyronine deiodinase (ITH-D) isozymes in thyroid hormone responsive tissues (Fig.1). These reductive deiodination reactions yield either the thyromimetically more potent 3,3',5-triiodo-L-thyronine, T3) or its isomer 'reverse T3' (3,3',5'-triiodo-L-thyronine, rT3) which may have regulatory importance in thyroid hormone metabolism. Meanwhile three ITH-D isozymes have been described differing in their physiological and biochemical characteristics. Under in vitro conditions the type I ITH-D isozyme is able to catalyze both 5'(3')- and 5(3)-deiodination reactions - depending on pH of incubation and the type of substrate offered (for review:/1/.

We have recently reported that type I ITH-D is inhibited by extracts and secondary metabolites of medicinal plants /2/. Some of their constituents have been isolated (/2/, for review see /3/) but the 'active principles' are not yet fully defined. Various extracts from the species Lycopus, Lithospermum and Melissa which have longstanding use as folk remedies have been shown to exhibit various antihormonal properties in vivo and in vitro. The regulatory feedback system of the axis pituitary-thyroid-periphery is affected at several levels by these crude plant extracts. Therefore we investigated the effects of well known constituents of these plants, as well as of related compounds on the interaction between thyroid stimulating hormone (TSH) and its receptor at the level of the thyroid /4/, as well as the inhibitory effect of these compounds on extrathyroidal deiodinative metabolism of iodothyronines.

The analysis of structure-activity relationships (SAR) of low molecular weight phenolic ITH-D inhibitors, especially various types of flavonoids in this study revealed a minimum essential inhibitor structure which could be

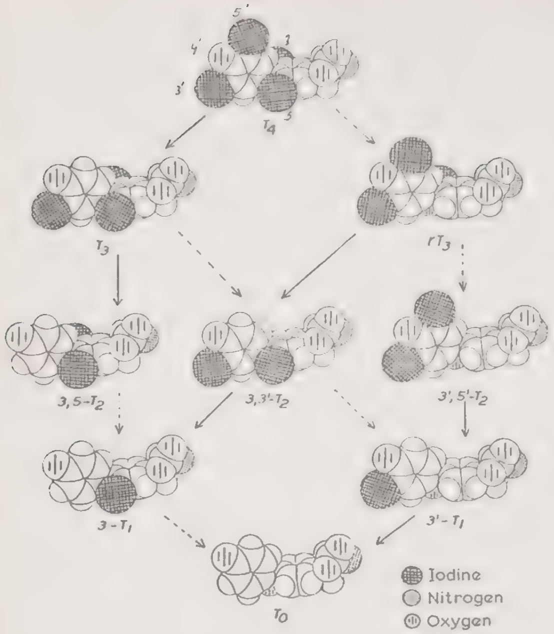

Fig.1 Thyroxine (T4) monodeiodination reactions are catalyzed by type I iodothyronine deiodinase in rat liver microsomal fractions. Solid arrows represent 5'(3')-deiodination reactions at the phenolic ring, broken arrows indicate 5(3)-deiodination at the tyrosyl ring of iodothyronines.

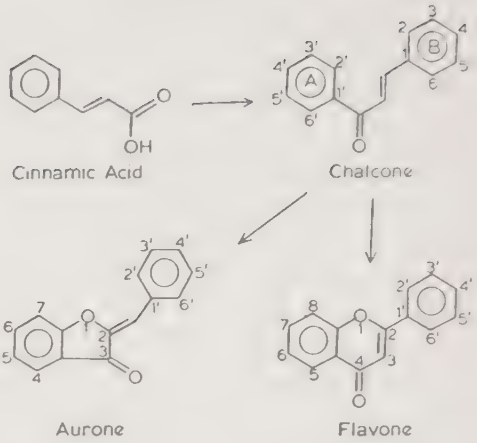

Fig.2 Classification and numbering scheme for flavonoids.

further optimized. Furthermore, a model of the ligand binding site of rat liver ITH-D can be developped. Computer graphic molecular modelling of both potent ITH-D inhibitors and of the complementary ITH-D ligand binding site may allow to design a new type of antithyroidal drugs based on extrathyroidal mechanism of action in contrast to thyreostatica with thyroidal mechanism of action.

MATERIALS AND METHODS

Iodothyronines were obtained by Dr. H. Rokos, Henning Berlin, GFR. Phenolic secondary metabolites of plants and other pure compounds were purchased from Roth, Karlsruhe, FRG; Sigma, München, FRG; Fluka, Buchs, CH; and Merck, Darmstadt, FRG. The aurones #42-44 were synthesized by Drs. Prücher and Irmscher, Merck, Darmstadt, FRG. Chalcones #10-12 and aurones #36-41 were kindly donated by the late Prof. E.C. Jorgensen, San Francisco, USA. Phloretin-4'-phosphate and PPP were a gift from Dr. H.Fex, AB Leo, Helsingborg, Sweden.

Rat liver microsomes were prepared and characterized as previously describe /5/. Rat hepatocytes were isolated by collagenase perfusion and incubated with ITHs in albumin-free Krebs-Ringer-bicarbonate buffer or Seglen's medium /6/.

ITH-deiodinase assays in rat liver microsomal fractions: Inhibition of various routes of the T4-monodeiodination cascade catalyzed by rat liver microsomal type I ITH-D was investigated. T4-5'-D, producing T3, was assayed at pH 7.4 /5/. rT3-production by T4-5-deiodinase was estimated either directly at pH 8.5 or via the fast indicator reaction producing 3,3'-T2 from T4 via rT3 at pH 7.4 /5/. Furthermore, 5-deiodination was monitored by a technique recently developped by us using a T4-analogue substrate which is selectively 5-deiodinated to a stable rT3-analogue which crossreacts with rT3-antiserum and can thus be quantified in a rT3-RIA /7/. For some compounds also the inhibition of rT3-5'-D was monitored using a radiometric assay which accounts for enzymic release of 125-I from 125-I-labelled rT3 /8/. Data for half-maximal inhibition of ITH-D are taken from semilogarithmic dose response curves of at least two closely agreeing experiments using at least 3, mostly 5 inhibitor concentrations ranging over two orders of magnitude. Inhibitors were dissolved either in double-distilled water, alkaline solution or methanol, if necessary.

Structural analysis and computer graphic modelling. Thyroid hormone conformations derived from crystallographic data /9,10/ were compared by the MMS-X computer graphic system (developped at Washington University, St. Louis,USA) with structural models for flavonoids and phenolic compounds using the crystal structures of phlorizin /11/, 4'-methoxy-aurone /12/ and other flavonoids /13/.

RESULTS

Table 1: T4-5'-DEIODINASE INHIBITION BY NATURALLY OCCURRING PHENOLIC SECONDARY METABOLITES OF PLANTS

SUBSTANCE	ID-50% microsomes
1. Luteolin-(7b-glycoside)	5 µM
2. rosmarinic acid	20 µM
3. ellagic acid	40 µM
4. (±) usnic acid	15-25 µM
5. esculetin	25 µM
6. (±) catechin	500 µM
7. (±) epicatechin	500 µM
# Lithospermum off., freeze-dried extract (100 C)-ether	5 µg/ml
8. humic acid	20 µg/ml
9. tannic acid	7 µg/ml

Cinnamic, (dihydro-)caffeic, ferulic, chlorogenic, sinapic acids; 4-OH- and 7-OH-coumarin; 2,3-; 2,4-, and 3,5-dihydroxy-benzoic acids were all inactive in concentrations below 100 uM.

Effects of single ring aromatic compounds on ITH-D. Phenolic plant goitrogens identified in legumes /14/, their synthetic analogues , triazol goitrogens, as well as precursors or metabolites of flavonoids derived from cinnamic acid are inactive as inhibitors in concentrations below 100 µM. Among those inactive compounds are a) the goitrogens 3,4-dihydroxy-, 2,4-dihydroxy-, 2,3-dihydroxy-pyridine; 3-hydroxypyrone; pyrazol; 3- and 4-amino-1,2,4-triazole; and b) phenolic seondary metabolites of plants : resorcinol, phloretic acid, phloroglucin, salicylate, the compounds mentioned in Table 1 and many other related phenols.

Effects of coumarins and related compounds on ITH-D. 4-OH- and 7-OH-Coumarin are inactive in contrast to esculetin, which reveals half-maximal T4-5'-D inhibition at 25 µM concentration. Both isomers (±) of the antibiotically active usnic acid, ubiquitous in lichen forms /15/, exhibit considerable potency (ID-50% 15 µM). Biopolymeric microbial degradation or auto-oxidation products of phenolic secondary metabolites of plants, e.g. humic and tannic acid exhibit a high inhibitory potency in the T4-5'-D reaction, which is comparable on a weight basis to the most active freeze dried extract of Lithospermum off.(ether extraction at pH 2 of a crude aqueous extract, 2h, 100 C): Lith.off FDE -e /2/.

Table 2: T4-5'-DEIODINASE INHIBITION BY (DIHYDRO-)CHALCONES AND RELATED COMPOUNDS

		substituents (other than H)							ID-50% (µM)	
		A-ring			B-ring		bridge		micro-somes	hepato-cytes
	chalcones	2'	4'	6'	3	4	7	8		
10.	2',4'-$(OH)_2$-	OH				OH			>100	
11.	2',4',4-$(OH)_3$-	OH	OH			OH			>100	
12.	2',4'-$(OH)_2$-3,4-methylenedioxy-	OH	OH		O-CH$_2$-O				>100	
13.	2',4',6',3,4-$(OH)_5$-	OH	OH	OH	OH	OH			8	
14.	2',6',4-$(OH)_3$-4'-methoxy-	OH	OCH$_3$	OH		OH			4	
	dihydrochalcones									
15.	phloretin	OH	OH	OH		OH	CH-CH		2.5	2.5
16.	phloretin-4-phosphate	OH	OH	OH		PO$_4$	CH-CH		9	
17.	phlorizin	OH	OH	O-gluc		OH	CH-CH		>100	
	related compounds									
18.	polyphloretin-phosphate (PPP)								0.2-0.5 µg/ml	
19.	phloroglucin	OH	OH	OH	-------	-------	------		>100	
20.	phloretic acid	-------	-------	-------		OH	-----		>100	

Effects of cinnamic acid derivatives consisting of at least two phenolic rings: (dihydro-)chalcones and related compounds. We have previously reported that dimers and polymers from cinnamic acid, e.g. rosmarinic acid and ellagic acid are potent ITH-D inhibitors /2/. Table 2 summarizes the inhibition data for some more naturally occurring secondary metabolites of plants of this type and for some synthetic analogues. The essential structural element necessary for inhibition of ITH-D seems to be a 2',4',6'-trisubstituted aromatic ring connected *via* a flexible bridge structure of varying chain length to a second phenolic ring with a negative charge in 4-position. The 4'-postion of ring A can be blocked without drastic loss in inhibitory potency (#14), however both a free 2'- *and* 6'-OH group are essential (#11,#14,#15,#17) (Tab.2). The phloretin biodegradation products phloroglucin and phloretic acid , representing the two separate essential phenolic rings, are each inactive in contrast to the highly potent chemically not defined phosphorylated phloretin polymer PPP. The olefinic character of the exocyclic C7-C8-chalcone bond is not essential as dihydrochalcones exhibiting the above mentioned characteristics (#15,#16) are also inhibitory active.

Table 3: T4-5'-DEIODINASE INHIBITION BY AURONES

aurones		substituents (other than H)									ID-50% (μM)	
		A-ring 4	6	7	B-ring 2'	3'	4'	5'	bridge 2	10	micro-somes	hepato-cytes
36. aureusidin	n	OH	OH			OH	OH				1	>10
37. trihydroxyaurone	n	OH	OH				OH				1.5	
38. 4'-acetoxyaurone	s	OH	OH				OAc				5	
39. 4'-carboxyaurone	s	OH	OH				COOH				>100	
40. 3'-iodo-4,4',6-$(OH)_3$-aurone	s	OH	OH			I	OH				0.5	0.5
41. 2,10-dihydro-aureusidin	s	OH	OH			OH	OH		CH-CH		8	
42. 10-methyl-aureusidin	s	OH	OH			OH	OH			CH_3	5	
43. 2',4,4',6-$(OH)_4$-aurone	s	OH	OH		OH		OH				1.5	5
44. 2',4,4',6-$(OH)_4$-3',5'-$(Br)_2$ aurone	s	OH	OH		OH	Br	OH	Br			1.5	5
45. sulfuretin	n		OH			OH	OH				8	
46. maritimetin	n		OH	OH		OH	OH				n.d.	
47. maritimein	n		O-gluc	OH		OH	OH				>100	

n: naturally occurring; s: synthetic

Effects of cinnamic acid derivatives with a rigid 5-membered heterocyclic bridge structure: Aurones. Naturally occurring aurones are the most potent T4-5'-D inhibitors among all phenolic secondary metabolits of plants tested so far and reach ID-50% values comparable to the apparent K_M-value of the hormone substrate T4 (2μM). The following SAR for aurones were found. The presence of a 3'-OH-group does not increase potency (#36,#37, Tab.3), the 4'-OH-function can be replaced by O-acetoxy- but not by a carboxyl-group (#37-39). Exchange of 3'- and 2'-OH-groups does not alter the potency (#36,#43). A 4-OH-group is essential for activity (#36,#45). Glycosylation in 6-position inactivates the compound (#47). Introduction of a methyl group in position 10 at the bridge structure reduces the inhibitory potency similar as reduction of the olefinic bond. Bromination in 3'- and 5'-position does not increase the potency compared to considerable activation by 3'-iodination (#42) which results in the most potent T4-5'-D inhibitor found so far among the naturally occurring secondary metabolites of plants and their synthetic derivatives.

Effects of inhibitors on the other reactions of the microsomal T4-monodeiodination cascade and on the T-5'-D of intact rat hepatocytes. Effective inhibitors act on all pathways of the cascade, i.e. SAR and inhibitory potency of representative chalcones and aurones are identical for T4-5'-, T4-5-, rT3-5'-, and T4-analogue-5-D reactions in the rat liver microsomal system.
In hepatocytes aureusidin did not inhibit T3-formation in 10 μM concentration in contrast to 3'-iodo-4,4',6-trihyroxy-aurone which revealed an inhibitory potency comparable to that in the microsomal membrane fraction: 0.5μM. Phloretin, the most potent chalcone was equally potent in hepatocytes and in the microsomal fraction. All other aurones and flavonoids tested so far are by a factor of 5-10 less active in the intact cells compared to the microsomal membranes /16; unpublished results, M.Spanka/.

Computer graphic modelling of flavonoid structures and structural comparison with iodothyronine conformations. Analysis of crystallograhpic data of naturally occurring ITHs revealed as a predominant structure the skewed ITH-conformation represented in Fig.1 and 3. The orientation of the phenolic ring plane of the molecules is perpendicular to and bisecting the tyrosyl ring plane, because the rotational freedom around the diphenylether bridge is hindered by the bulky iodine atoms in 3- and 5-position /9/. For rT3 a second conformation, antiskewed, was found recently /10/. The removal of the 5-iodine atom at the tyrosyl ring enables a 'relaxed conformation' where the orientation of the tyrosyl ring plane is perpendicular to and bisecting the plane of the phenolic ring (Fig.3, middle). Most of the structures reported so far [illegible] flavonoids show coplanar orientation of the two phenolic rings, however also skewed conformations are known /11-13/. In contrast to the ITH structures the rotational freedom between the two phenolic rings of (dihydro-)chalcones, aurones and other flavonoids is not hindered. Therefore we tried to match possible flavonoid structures to known ITH-conformations (Fig.3). The two phenolic rings of the ITHs and the aurone, respectively dihydrochalcone, were taken as fixed structure for overlap because 3'-iodination of aureusidin increased inhibitory potency considerably (Tab.3). Therefore the A-ring of chalcones or the benzopyrone or benzofuran ring of the flavonoids overlaps with the tyrosyl ring of ITHs (Figs.3,4). In this orientation the functional groups of flavonoids closely match essential polar residues of the ITHs which are necessary for optimal ligand enzyme interaction (5,16; unpublished results, Köhrle). Surprisingly, the most potent dihydrochalcone and the 3'-iodo-4,4',6-trihydroxy-aurone can mimic the antiskewed structure of rT3 as illustrated in the difference map of the van der Waals electron densities of both molecules (Fig.4). rT3 is the substrate with the highest affinity in the type I ITH-D reaction and the most potent competitive inhibitor of the T4-5'-D-reaction. Therefore the close structural and conformational similarity between the antiskewed rT3 structure and the model conformations of potent phenolic ITH-D inhibitors could be an explanation for the high inhibitory potency of these two compounds both in the ITH-D-reactions of the microsomal system and of intact hepatocytes.

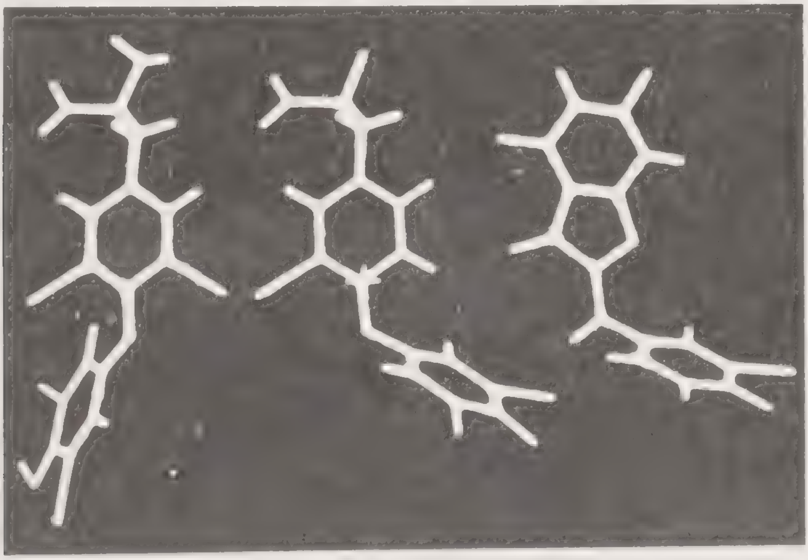

Fig.3 Computer graphic comparison of the skewed conformation of L-thyroxine (T4) (left), the antiskewed conformation of rT3 (middle), and the antiskewed conformation of 3'-iodo-4,4',6-trihydroxy-aurone (right).

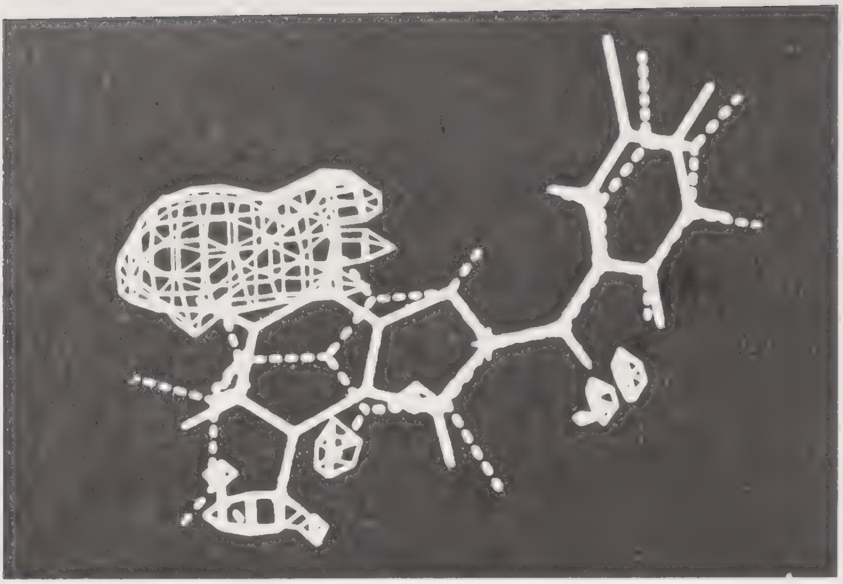

Fig.4 Computer graphic presentation of the difference map of the van der Waals electron densities of 3'-iodo-4,4',6-trihydroxy-aurone (solid structure) and the antiskewed rT3 (dotted structure). The 'chicken-wire grid' illustrates the extra conformational space occupied by the aurone in comparison to rT3.

DISCUSSION

T4- and iodothyronine -deiodination, catalyzed by type I ITH-D in rat liver microsomal fractions and intact hepatocytes, is inhibited by phenolic secondary metabolites of plants with halfmaximal inhibitor concentrations equal or even lower than the apparent Michaelis-Menten constants for the enzyme substrates. Therefore the low molecular weight phenolic secondary metabolites of plants may represent one antithyroidal principle contained in medicinal plants with longstanding use in folk medicine. This principle seems to be different from other actions of these plant extracts on the pituitary-thyroid axis described previously, e.g. inactivation of TSH by oxidized cinnamic acid derivatives which form adducts with TSH making the hormone unable to bind to the TSH-receptor and to stimulate thereby cyclic-AMP production /4/. Furthermore various effects on thyroid hormone biosynthesis and antigonadotropic effects were reported /for review see 2,4/.

The analysis of SAR for phenolic inhibitors of ITH-D enables us to derive minimal essential inhibitory structures (Fig.5) and to optimize these compounds in order to increase their potency and selectivity for ITH-D inhibition for potential development of new types of antithyroidal drugs. Single-ring phenolic compounds, among them well known goitrogens /14/, are inactive as ITH-D inhibitors though they contain the structural elements of potent inhibitors. The latter, however, consist of at least two phenolic rings connected by a bridge

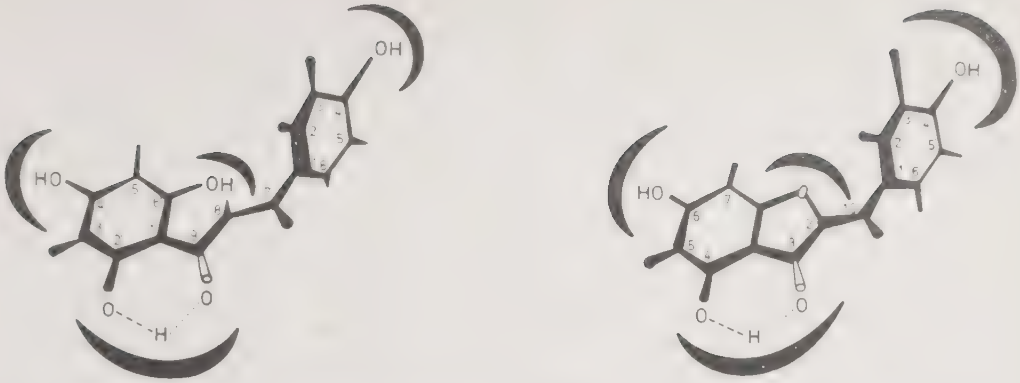

Fig.5 Models of the minimal essential inhibitor structures of chalcones (left) and aurones (right) and illustration of the corresponding pockets of ligand binding site of the iodothyronine deiodinase

structure of varying constitution and length (depside, (un-)saturated hydrocarbon chain, five- or six-memberd O-heterocycle). Inhibitory active compounds of this type exhibit remarkable conformational similarity to the antiskewed structure of rT3 - the most potent physiologically occurring inhibitor of rat liver type I ITH-D. Fig. 4 illustrates the close similarity of the three-dimensional structures of rT3 and a model conformation of 3'-iodo,4,4',6-trihydroxy-aurone. Both phenolic rings are almost completely overlapped; the diphenylether structure of the ITH is similar to the exocyclic olefinic bond of the aurone and the essential4-OH-group and the 6-OH-group of the aurone point into the direction occupied by the side chain of the ITH. We have previously shown, that ITH-analogues exhibit optimal ligand properties if the side chain contains no positive charge and has only short chain length (e.g. acetic acid) /5,16/. Therefore the 6-OH-function could mimic this essential part of the ITH-molecule during ligand-enzyme interaction. Presumably the glycosyl-residue in maritimein may prevent this favourable interaction.

The analysis of the SAR for ITH-D inhibitors both of the aurone and the (dihydro-)chalcone class revealed minimal essential inhibitor structures depicted in Fig.5: two phenolic ring systems connected by a rather rigid bridge structure in a way that the orientation of the two phenolic ring planes is mutually perpendicular and that two phenolic groups are exposed at both distal portions of the molecule. Furthermore an intramolecular hydrogen bond or metal cation chelating arrangement should be possible between the carbonyl function of the heterocycle or bridge structure and the hydroxyl group in direct neighbourhood (4-OH in aurones, 2'-OH in chalcones).

On the basis of this minimal essential inhibitor structure for the aurones we tried to optimize the inhibitors by chemical modification. Producing a negative charge in 4'-position (#38,#39) we found that a formic acid derivative lost its inhibitory potency. This is similar to a previous finding that T4-formic acid is not a substrate for ITH-5'-D in contrast to the optimal T4-acetic acid /5/. This result for the substrate could explain why O-acetoxy-aurone is a rather potent compound. Presumably the negative side chain charge of this inhibitor is at a similar optimal distance from the phenolic ring as that of the acetic acid in the T4-analogue substrate. Highest inhibitory potency was reached by the 3'-iodination which both acidifies the phenolic 4'-OH-group and also introduces iodine into the molecule. We have previously found, that iodination of ITH-D ligands leads to a considerable increase in ligand affinity in contrast to introduction of other halogen atoms, e.g. bromine as in the aurone derivative #44. Presumably the deiodinase is a member of a class of highly specialyzed proteins which preferentially bind iodinated, phenolic single- or poly-cyclic (aromatic) ring systems such as iodophenols, iodinated X-ray contrast agents, and iodothyronines, their 'original' ligands /5,7,16/.

The third approach for optimization of the inhibitory active aurones was concentrated on the bridge structure between the two phenolic ring systems. If our model of conformational similarity between ITH-D substrates and inhibitory phenolic ligands is correct, then a fixation of the antiskewed or skewed conformation of the inhibitor molecules should increase inhibitory potency by generation of a 'conformationally frozen' molecule. Both of these approaches, hydroxylation in 2'-position (#43) and methylation in 10-position (#42) did not increase (#43) or even decreased (#42) inhibitory potency, although these modifications result in a limitation of the rotational freedom between the two aromatic rings due to the introduction of bulky substituents. However, the reduction of the exocyclic olefinic bond (#41) which releases conformational constraint of the aurones also leads to a decreased potency.

The fact that the 10-methyl-aurone is still a potent inhibitor excludes the involvement of the acidic hydrogen in 10-position of the natural aurones in the inhibitory mechanism. Up to now we also have no evidence for covalent interaction of the aurone inhibitors with the enzyme, which could result from such a type of reaction. Therefore we conclude that introduction of conformational rigidity does not increase the inhibitory potency of aurones beyond that already reached by the naturally occuring 'Leitsubstanz' aureusidin. Only iodination of the phenolic ring increases the inhibitory potency. A definite answer on the role of the aurone conformation for inhibitory potency will be possible after the analysis of the X-ray structure of the molecules which is currently under investigation.

Further modifications of the 'Leitsubstanz' at the benzofuran-ring will be performed by computer graphic analysis studies based on two models of potent ITH-D ligands: the model described here for phenolic secondary metabolites of plants and their synthetic analogues, and another equivalent model, based on variations of the ITH-substrate structure, which leads to optimized ITH-D substrates and inhibitory active ITH-analogues /5,7,16/. The combination of both approaches will enable us to design a complementary model of the ITH-D ligand binding site for 'antithyroidal drug design' and optimization. Furthermore, the development of active site-directed affinity ligands for the type I ITH-D will be possible for further characterization of the enzyme reaction and purification of the key enzyme of the iodothyronine metabolism, the iodothyronine deiodinase. --- Supported by research grants from Deutsche Forschungsgemeinschaft and NIH.

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ROLE OF FLAVONOIDS IN LIPOPEROXIDATION AND RADICALAR REACTIONS

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INTRODUCTION

Flavonoids are generally considered to be antioxidants. We demonstrated several years ago that the expression "antioxidant" is indefinite, unspecific, and must be replaced by one of the three expressions: radical scavenger or antiradicalar agent, antilipoperoxidant, and antioxygen (1), according to the properties of the given substance. It is a misconception to accosiate necessarily the antiradicalar, antilipoperoxidant and antioxygen activities.

Antiradicalar activity is estimated by the ability of a substance to scavenge free radicals (OH·, DPPH, ...).

Antilipoperoxidant action is the capacity of substances to interrupt a chemical autoxidation cycle initiated by lipoperoxides in unsaturated fats. Antioxygen agents are substances which compete with others to bind triplet oxygen. These distinctions are not captious: for instance, we demonstrated that antilipoperoxidants inhibit cyclooxygenase (which synthesizes prostaglandins), while antiradicalar agents stimulate this enzyme (2).

In this paper we try to determine antiradicalar, antilipoperoxidant and antioxygen activities of several flavonoids, with our methods (3, 4). Quercetin, rutin, hesperidin, kaempferol, and an extract of Ginkgo biloba (GBE) were investigated. Our results were also confirmed by assays on cyclooxygenase activity. Uric acid, a potent ·OH radical scavenger (5, 6), was used as comparative reference.

METHODS

1. Reagents

Rutin, kamepferol and quercetin (Merck), hesperidin (Eastman Kodak) were dissolved in dilute alkaline solutions and added to the different systems described further, in final concentrations of 10^{-4} and 10^{-5} M in phosphate buffer (pH 7.4). A Ginkgo biloba extract (Institut H. Beaufour) was used, which contained flavonoid heterosides and proanthocyanidins; the mean molecular weight of these flavonoids was approximately 720. The concentrations of Ginkgo extracts used were 125, 250 and 500 µg/ml. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Aldrich. Hyaluronic acid was a Sigma product.

2. Antiradicalar properties

Hydroxyl radical was produced by γ-irradiation (10 Gy; ^{137}Cs; 0.8 Gy/min) in a solution 10^{-3} M of 2-keto-4-methylthiobutyric acid(KMB - Sigma), under N_2O, in phosphate buffer (pH 7.4); ethylene, formed by reaction between $^{\cdot}OH$ and KMB (7), was measured by gas-liquid chromatography, using a Porapak column in a Barber-Colman 3000 gas chromatograph, equipped with FID (temperatures: column, 100° C; injector, 160° C; FID, 180° C), using argon as mobile phase.

Hydroxyl radical was also radioinduced (same conditions as above) in hyaluronic acid (HYA : 1.6 mg/ml in phosphate buffer, pH : 7.4), under N_2O (6); $^{\cdot}OH$ decreased HYA viscosity, by depolymerization. Viscosimetry was measured by an Ostwald viscosimeter (6).

Hydroxyl radical was also produced by Fenton reaction: ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$; $FeSO_4$: 10^{-4}; H_2O_2 : 10^{-4}; EDTA : 5.10^{-5} M), in a solution of 5,5-dimethyl-1-pyrroline N-oxide (DMPO : 10^{-3} M). $^{\cdot}OH$ radical reacted with DMPO to give a long-lived radical spin adduct (DMPOOH), which was easily identified by ESR spectrometry (8) on a Varian E-9-X Band (9.5 GHz) spectrometer (field modulation : 100 kHz).

Antiradicalar potencies of flavonoids were also tested against the solution of diphenylpicrylhydrazyl (DPPH), a stable free radical; the scavenging efficiency was measured by DPPH discoloration at 517 nm (9).

3. Antilipoperoxidant properties

A chemical fatty acid autoxidation cycle was maintained by the incubation of a determined quantity of linoleic acid hydroperoxide, in the presence of non-peroxidized arachidonic acid. Ferrous ion (10^{-4} M) was added to the system, together with EDTA (5.10^{-5} M) as ligand, in order to reactivate the cycle (3).

Reactions were performed in sealed vials filled with pure oxygen. Lipoperoxides react with Fe^{2+} to give alkoxy radicals. These radicals are able to remove hydrogen atoms from intact molecules of arachidonate, which become, in their turn, radicalar and able to react with oxygen; peroxy radicals are so formed, which can remove hydrogen atoms from new arachidonate molecules, etc.

After 20 and 30 minutes, lipoperoxides were estimated by the DMPD (N,N′-dimethyl-p-phenylenediamine) method, previously described (3). Liver microsomes-induced peroxidations were performed as described by Ernster (10). The mixture was composed of 125 mM KCl, 25 mM Tris, pH 7.4, and 1 mg protein per ml of microsomes. Lipid peroxidation has been induced by the following system: 0.18 mM NADPH, 12 μM $FeCl_3$ and 1 mM ADP. Flavonoids were added two minutes before the addition of the peroxidizing system. Reaction was carried out for 30 minutes at 25° C with constant shaking. Malonaldehyde (MDA) was determined by the thiobarbituric acid method (11).

4. Antioxygen activity

Ascorbate (AH_2) is oxidized by molecular oxygen, forming dehydroascorbate (DHA), the reaction being favoured in alkaline medium. The rate of inhibition of DHA formation, at pH 8.0, was taken as an index of antioxygen activity.

176 mg AH_2 was dissolved in 10 ml NaOH 0.1 N, the pH being 5.7. This solution was progressively alkalinized by addi-

tion of small volumes of NaOH 0.1 N, in the presence of dissolved oxygen, at room temperature. The formation of DHA was followed by spectrophotometry, at 700 nm, after 30 minutes.

5. Effects on cyclooxygenase

Cyclooxygenase activity was studied using microsomes of bull seminal vesicles, according to a previously described technique (5).

Briefly, labelled arachidonate (5.10^{-6} M) was incubated with microsomes, in the presence of glutathione (10^{-4} M); labelled products were extracted by diethyl ether, chromatographed on TLC plates, eluted and counted in a beta spectrometer. Effects of flavonoids were determined by comparison with controls.

RESULTS

1. Antiradicalar properties

Spin-adduct formation from DMPO by $^{\bullet}$OH was followed by measuring the second ray of the DMPO-OH spectrum in ESR spectrometry. Inhibition of $^{\bullet}$OH formation was expressed by the reduction of the 2nd ray amplitude. Table I shows the values obtained with flavonoids, and compared with uric acid at 10^{-4} M. The order of efficiency of scavengers was: GBE (250 µg) > rutin > uric acid > quercetin; only a weak effect was observed for kaempferol and hesperidin.

Table II shows the results obtained on ethylene production by irradiated solution of KMB. At 10^{-3} M, uric acid was the most efficient agent followed by rutin > quercetin > kaempferol > hesperidin. At 10^{-4} M, rutin exerted a higher inhibitory action than uric acid. Hesperidin was a particular case, because at lower concentrations, it enhanced $^{\bullet}$OH production. GBE exerted a marked effect at the three concentrations tested. At 250 µg/ml, it exhibited approximately the same inhibiting effect as quercetin at 10^{-3}, or rutin at 10^{-4}.

Effects of flavonoids and uric acid on HYA depolymerization are shown in Table III. The order of efficiency to inhibit

Table I: Inhibition by flavonoids of the 2nd ray of the DMPO-OH spectrum

	% inhibition after 5 min
Control	0
Quercetin 10^{-4} M	19
Rutin 10^{-4} M	40
Hesperidin 10^{-4} M	15
Kaempferol 10^{-4} M	8
Uric acid 10^{-4} M	24
GBE 250 µg/ml	60

Table II: Modification by flavonoids of ethylene produced by γ-irradiation of KMB solution (expressed in % of control)

	10^{-3}M	10^{-4} M	10^{-5} M	125 µg/ml	250 µg/ml	500 µg/ml
Control	100	100	100	100	100	100
Uric acid	14	66	89			
Rutin	29	56	96			
Quercetin	56	76	91			
Hesperidin	91	111	122			
Kaempferol	75	93	98			
GBE				65	51	37

viscosimetry decrease was at 10^{-4} M as follows: rutin > uric acid > kaempferol = quercetin = hesperidin. At 125 µg/ml, GBE was as efficient as uric acid (10^{-4} M).

Table IV shows the efficiency of the different agents to destroy DPPH. At 10^{-4} M, the order of efficiency was: quercetin = uric acid > rutin = kaempferol > hesperidin. GBE, at 250 µg/ml, exerted approximately the same effect as quercetin at 10^{-4} M.

Table III: Protective effect of flavonoids on the viscosity decrease of a hyaluronic acid solution (HYA) irradiated by γ-rays (^{137}Cs)

	Viscosity of HYA	
	non irradiated	irradiated
None	100 %	37 %
Quercetin 10^{-4} M	97 %	42 %
Rutin 10^{-4} M	100 %	58 %
Hesperidin 10^{-4} M	100 %	42 %
Kaempferol 10^{-4} M	100 %	42 %
GBE 125 µg/ml	97 %	49 %
Uric acid 10^{-4} M	100 %	49 %

Table IV: DPPH radical destruction by flavonoids (expressed in % of control)

	10^{-3} M	10^{-4} M	10^{-5} M	125 µg/ml	250 µg/ml	500 µg/ml
None	0	0	0	0	0	0
Quercetin	100	100	4			
Rutin	100	32	3			
Hesperidin	100	11	0			
Kaempferol	100	28	0			
GBE				44	100	100
Uric acid		100	72			

2. Antilipoperoxidant activities

The capacity of the studied substances to interrupt a chemical fatty acid autoxidation cycle initiated by a given amount of lipoperoxides is represented in Table V.

Results were compared to those obtained with a classical antilipoperoxidant: nor-dihydroguaiaretic acid (NDGA). Measurements were performed after 20 min. It clearly appears that the

Table V: Effect of flavonoids on a chemical fatty acid autoxidation cycle (antilipoperoxidant power)

	% of control
Quercetin 10^{-3} M	75
Rutin 10^{-3} M	98
Hesperidin 10^{-3} M	131
Kaempferol 10^{-3} M	105
GBE 250 µg/ml	92
GBE 125 µg/ml	94
Uric acid 10^{-3} M	100
NDGA 10^{-3} M	0

Table VI: Effect of flavonoids on NADPH-dependent microsomal peroxidation

	% malonaldehyde formation
Control	100
Quercetin 10^{-4} M	0
Quercetin 10^{-5} M	43
Rutin 10^{-4} M	73
Rutin 10^{-5} M	94
Hesperidin 10^{-4} M	100
Hesperidin 10^{-5} M	121
GBE 500 µg/ml	0
GBE 250 µg/ml	0
GBE 125 µg/ml	0

different reagents are weak antilipoperoxidants, or, even, prolipoperoxidants, as it is the case for hesperidin.

The results are quite different in the case of lipoperoxidation induced by liver microsomes (Table VI). At 10^{-4} M, quercetin inhibited completely the MDA production. Rutin effect was much weaker, while hesperidin behaved as a prolipoperoxidant, particularly at 10^{-5} M. GBE was inhibitor, even at 125 µg/ml.

Table VII: Effect of flavonoids on ascorbic acid autoxidation (antioxygen power)

		µg/ml dehydroascorbic acid after 30 min
Ascorbic acid (pH 5.7)		1.1
Ascorbic acid (pH 8.0)		14.9
	+ Quercetin 10^{-3} M	8.8
	+ Rutin 10^{-3} M	9.3
	+ Kaempferol 10^{-3} M	12.3
	+ Hesperidin 10^{-3} M	16.0
	+ GBE 250 µg/ml	11.1
	+ GBE 125 µg/ml	11.9

Table VIII: Effects of flavonoids on cyclooxygenase activity (expressed in % of control)

	10^{-3} M	10^{-4} M	10^{-5} M	125 µg/ml	250 µg/ml	500 µg/ml
Quercetin	10	74	77			
Rutin	207	120	110			
Hesperidin	70	96	81			
Kaempferol	-	100	76			
GBE				120	140	140
Uric acid	200	140				
NDGA	8					

3. Antioxygen properties

The capacity of flavonoids to protect ascorbate against the oxidation of molecular oxygen is shown in Table VII. The potentiating effect of high pH on ascorbate oxidation appears clearly (1.1 µg/ml at pH 5.7; 14.9 µg/ml of DHA formed at pH 8.0). Rutin (10^{-3} M) and moreover quercetin (10^{-3} M) are able to decrease the DHA formation in an important way. Kaempferol and GBE have moderate effects. On the contrary, hesperidin seems to favour the autoxidation of ascorbic acid.

4. Cyclooxygenase modulation (Table VIII)

Hesperidin and kaempferol are without marked effects on cyclooxygenase activity. Rutin (10^{-4} M) and GBE are moderate activators, but at 10^{-3} M rutin exhibits an important stimulatory effect. Quercetin exerts an inhibition, which becomes significant at 10^{-4} M, and very strong at 10^{-3} M. Uric acid is a potent stimulator at 10^{-3} M. However, NDGA (10^{-3} M) has a powerful inhibitory effect.

DISCUSSION

It clearly appears from the different data exposed in this paper that the interrupting effect of autoxidation cycles is not closely associated to the antiradicalar action (anti-$^{\bullet}$OH or anti-DPPH).

1. Antiradicalar effects

Rutin behaves as an efficient $^{\bullet}$OH scavenger, in the three techniques employed in this investigation; its efficiency is near to that of uric acid, a potent hydroxyl scavenger (6). Quercetin exerts a weaker action on $^{\bullet}$OH radical, but is more efficient than rutin on DPPH destruction. This example is a good demonstration that the free radical scavenging activity of a given agent is dependent on the nature of the radical (2). This specificity can be explained by a stereochemical compatibility. Such compatibilities are evidenced by hesperidin and kaempferol, which are weak $^{\bullet}$OH radical scavengers, even at high concentrations, but strong DPPH destroyers. Moreover, at low concentration, hesperidin seems to be a stimulating agent on $^{\bullet}$OH production in irradiated solution. GBE behaves as a good free radical scavenger, acting as well on $^{\bullet}$OH as on DPPH.

2. Antilipoperoxidant effects

When assayed on a chemical lipoperoxidation cycle and compared to NDGA, flavonoids exhibit weak (quercetin and GBE) or no (rutin and kaempferol) inhibiting action. Moreover, hesperidin stimulates the rate of autoxidation. This unexpected property of hesperidin must be associated to its capacity to enhance $^{\cdot}OH$ production (see above), and can be explained by a complexing action on transition metals.

Thus, antiradicalar activity is not necessarily linked to the capacity of interrupting lipid autoxidation, during which though lipid radicals ($RO^{\cdot}$ and $ROO^{\cdot}$) are generated. Such dissociation was also observed for uric acid. The results are quite different when lipid peroxidation is induced in liver microsomes. In this complex kind of peroxidation, GBE and quercetin exhibit a strong inhibitory effect. It was demonstrated (8, 12) that $^{\cdot}OH$ radical is the oxidizing agent during microsome peroxidation. Hydroxyl radical is generated by a Fenton reaction involving Fe^{2+}, which originates from the reduction of Fe^{3+}, by a NADPH-dependent reductase, present in the system (10). Quercetin and GBE could inhibit the peroxidation by an $^{\cdot}OH$ scavenging action, which could be potentiated by the regeneration of the reduced form of the flavanoids by the NADPH-reductase. This reduction occurs when the redox potential of the studied flavonoid is adequate; it is not the case for rutin (which is although a powerful anti-$^{\cdot}OH$).

The stimulating action of hesperidin, at low concentration, observed on microsomes peroxidation, can always be explained by a ligand effect on transition metals.

3. Antioxygen effects

Antioxygen substances are characterized by a better capacity to react with triplet oxygen than others, which are protected in this way against oxidation or oxygenation.

Ascorbate was used to measure changes in oxygen reactivity induced by the introduction in the medium of various compounds. The decrease of ascorbate oxidation rate indicated the capacity of a given compound to react more rapidly with O_2, protecting the medium. Two compounds must be underlined: quercetin, which exerted the best antioxygen action, and hesperidin, which enhanced the ascorbate cxidation.

4. Cyclooxygenase modulation

Cyclooxygenase activity (CYAC) includes lipoperoxidation steps, and is thus inhibited by antilipoperoxidant agents. On the other hand, cyclooxygenase is auto-inactivated by ˙OH generated during CYAC (13). Thus, radical scavengers protect cyclooxygenase against ˙OH and enhance its activity. These antagonistic mechanisms are represented in Figure 1. When anti-

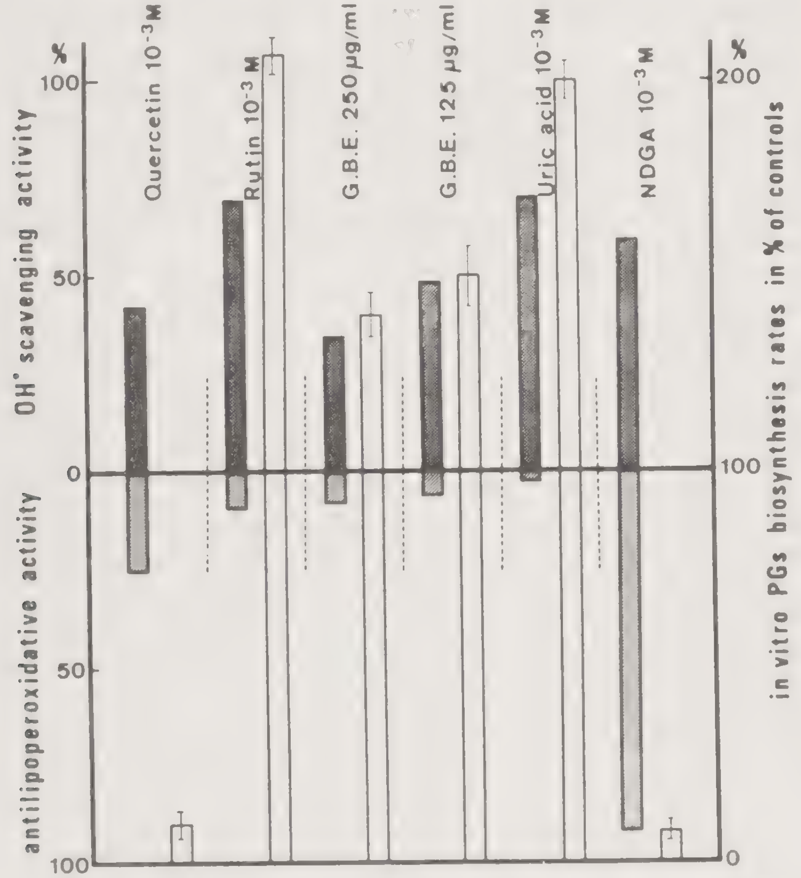

Fig. 1. Combined effects of antilipoperoxidant and antiradicalar activities of flavonoids on *in vitro* cyclooxygenase activity (bull seminal vesicles microsomes)

▨ OH˙ scavenging activity

▩ antilipoperoxidative activity

□ *in vitro* PGs biosynthesis rates in % or controls radicalar property is prevalent (as is the case for uric acid, GBE and rutin), CYAC is stimulated. When antilipoperoxidant effect is sufficiently marked (case of NDGA), the resulting action is an inhibition of CYAC. When the two effects are equilibrated, no influence is observed (kaempferol). The opposite actions are also equilibrated in the case of quercetin, but another phenomenon will intervene: the antioxygen property. Cyclooxygenase is a dioxygenase, using molecular oxygen for prostanoid synthesis. The substances able to compete for oxygen utilization will inhibit CYAC. Quercetin, as described above, is a good antioxygen agent; this property, added to the moderate antilipoperoxidant action, explains the potent inhibiting effect of this flavonoid on cyclooxygenase.

A stimulating effect could be expected for hesperidin, which is devoid of antioxygen property, and is prolipoperoxidant; however, this compound seems to increase $^{\cdot}OH$ generation (Table II). This last fact could explain the moderate inhibition observed with hesperidin.

CONCLUSION

This study on some flavonoids clearly shows the opportunity to distinguish at least three mechanisms, which are usually fused under the word antioxidant, and which are not necessarily associated. These distinctions allow to avoid the apparent confusion in the actions of "antioxidants" on biochemical phenomena, such as prostaglandin synthesis or microsomal peroxidation, which can be found in the previous literature.

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ALTERED PATTERNS OF DRUG METABOLISM IN LIVER DISEASE AND THEIR NORMALIZATION BY CYANIDANOL-3

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In recent years, attention has been given by my research group to the metabolism of both flavonoid and non-flavonoid drugs in the course of induced experimental diseases /1/2/3/4/. The galactosamine-hepatitis rat model, which has been shown to mirror the biochemical and histopathological changes observed in acute viral hepatitis in man /5/, was selected by us for investigations on the effect of an induced liver disease on the metabolism and excretion of certain drugs. Among the drugs investigated were the flavonoid compound, 3-O-methyl(+)catechin (1) and the non-flavonoid drugs: imipramine (2) and phenytoin (3).

Experimental hepatitis was induced by intraperitoneal administration of D-galactosamine (1g/kg body weight) to rats of approx. 250 g weight and the development of the hepatitis was monitored by determination of serum transaminase levels. Elevation of the SGPT levels (1) was observed in accord with the findings by Keppler et al. /5/.

The radiochemically-labelled drugs: 3-O-methyl[^{14}C](+)-catechin, [methylene ^{14}C]imipramine and [^{14}C]phenytoin were then separately administered orally to bile-duct cannulated rats, 18 h after injection of

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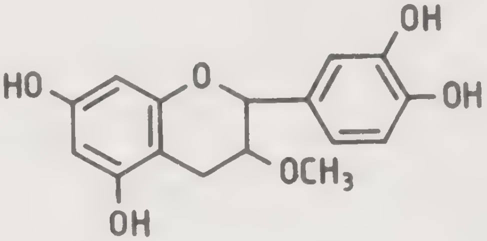
+Present address: Department of Metabolism and Pharmacokinetics, Huntingdon Research Centre, Huntingdon, UK of D-galactosamine, and bile and urine samples collected over 0-96 hours /2/3/. Similar collections were made from similarly cannulated control rats, untreated with D-galactosamine. The chromatographic procedures including h.p.l.c., t.l.c. and radioscanning have been previously described /2/3/4/6/.

Although the induction of galactosamine-hepatitis was shown to result in a number of changes in the metabolism of the drugs investigated, perhaps the most significant change observed was the marked depression in the biliary excretion of metabolites compared to those seen in the control rats.



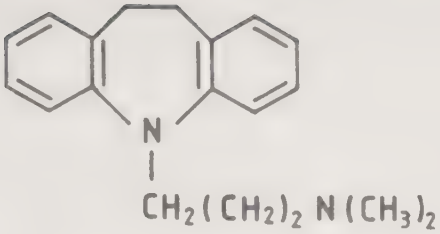
3-O̲-methyl (+) - catechin

(1̲)

The flavonoid,3-O̲-methyl(+)catechin (1̲), a specific histidine decarboxylase inhibitor, was orally administered to rats at a dosage level of 30 mg/kg body weight. The compound was shown to undergo restricted biliary excretion (33%) in galactosamine-hepatitis induced rats compared to 58% in control rats (2).

Isolation and identification of the biliary metabolites by h.p.l.c. and radioscanning procedures /2/ revealed the presence of the major metabolites, 3,3',-O̲-dimethyl(+)-catechin glucuronide and 3,3'-O̲-dimethyl(+) catechin sulphate which were previously detected in the bile of normal rats /6/.

The clearance of [^{14}C]labelled metabolites from plasma in galactosamine hepatitis was found to be prolonged. Faecal excretion of this compound over 96 h was correspondingly reduced from 44% in the normal rats to 4% in rats, in which experimental hepatitis had been induced.

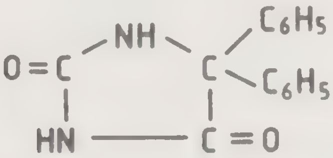


Imipramine

(2)

Investigations on the non-flavonoid drug [^{14}C]imipramine (2)/3/ revealed that excretion of its ^{14}C metabolites was also much impaired by the prior induction of galactosamine-hepatitis; biliary excretion following oral administration of imipramine (20 mg/kg body weight) being reduced from 80% to 35.5%. Faecal excretion of imipramin metabolites was lowered from 68% to 27% identified by h.p.l.c. and t.l.c. The major biliary metabolites of imipramine were 2-hydroxyimipramine-glucuronide, 2-hydroxydesmethyl-imipramine glucuronide and 10-hydroxyimipramine glucuronide. The two former metabolites were selectively reduced in quantity in bile from rats showing induced hepatitis.

Plasma clearance of imipramine after i.v. dosage was also decreased by 60%, whilst clearance of imipramine metabolites was reduced by 40% /3/.



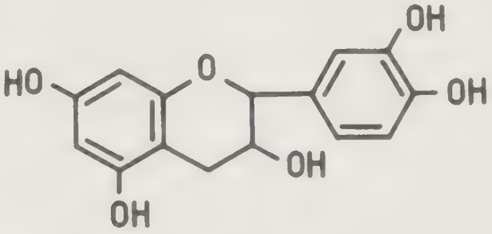
Phenytoin

(Diphenylhydantoin)

(3)

Administration of the anti-convulsant drug, phenytoin (3) to rats following the induction of hepatitis resulted in biliary excretion of only 39% of the oral dose in 24 h compared to 72.8% biliary excretion in the controls over the same period (Fig. 1). The major biliary metabolite in both cases was hydroxy-phenytoin glucuronide. In galactosamine-hepatitis, the plasma levels of [^{14}C]phenytoin were again elevated over the values seen in control rats (Fig. 2). In normal rats serum clearance of phenytoin was 0.20 ℓ/h. This was decreased in galactosamine-hepatitis to 0.90 ℓ/h.

It will be noted that all of these drugs, following the induction of galactosamine hepatitis, show a marked reduction in the biliary excretion of their metabolites and some degree of elevation of plasma levels of metabolites. Such reduced excretion and slow plasma clearance may well be associated with toxicity in those drugs, which are tolerated only within well defined dosage limits.



Cyanidanol - 3

(4)

Since the flavonoid drug, cyanidanol-3 (4) has been widely used in the treatment of acute viral hepatitis /7/ in man and is known to have hepato- protective properties in a number of experimental models /8/9/10/ it appeared of interest to establish the possible effects of this compound upon the depressed biliary excretion levels observed in galactosamine-hepatitis.

In these experiments a sub-chronic oral dosing regimen of cyanidanol-3 (at 0, 12, 24, 36 and 48 h) was employed /4/. The peritoneal administration of galactosamine was effected at 24 h, preceding the oral administration of [^{14}C]imipramine at 30 h. Bile duct cannulation was effected at 27 h.

The results obtained showed that cyanidanol-3 had largely restored normal levels of biliary excretion of imipramine in galactosamine hepatitis /4/. Incomplete restoration by cyanidanol may be due to cyanidanol molecules competing for the same mechanism of biliary excretion as imipramine, or alternatively, exerting an inhibitory effect upon those metabolic reactions in imipramine biotransformation which facilitate biliary excretion.

Cyanidanol was also observed to be effective in lowering plasma levels of imipramine in galactosamine hepatitis; a possible consequence of increased efficiency of biliary excretion.

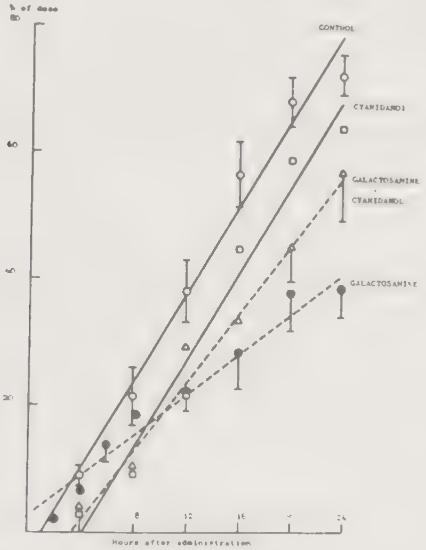


Fig. 1. The cumulative excretion of ^{14}C in the bile of the rat following oral administration of ^{14}C-phenytoin.

The ability of cyanidanol to restore normal levels of phenytoin excretion in bile was also explored. The dosage regimen and pre-treatment of rats receiving phenytoin was simlar to that employed in the imipramine studies but phenytoin was dosed at the level of 5.8 mg/kg body weight. Sub-chronic administration of cyanidanol was found to elevate biliary excretion of phenytoin from 34% in the galactosamine-treated rats to 57% in rats concurrently receiving cyanidanol. It will be noted that although complete restoration of biliary excretion has not been obtained over the 24 h period, nevertheless significant elevation is observed (Fig. 1).

Treatment with cyanidanol was also observed to lower the plasma levels of phenytoin, which are elevated in galactosamine-hepatitis (Fig. 2).

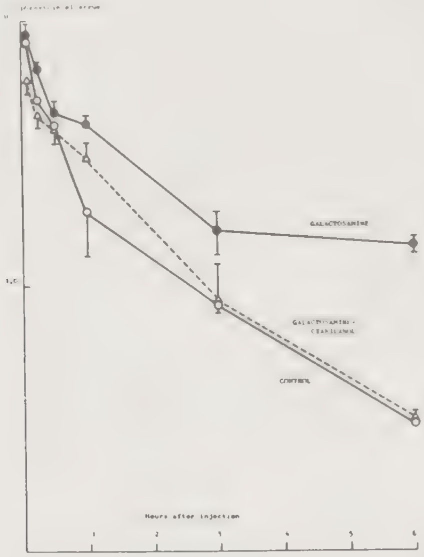


Fig. 2. The serum concentration of phenytoin following i.v. administration of this drug.

Although cyanidanol-3 has been shown to largely restore biliary excretion and normal plasma clearances of these two drugs in galactosamine- hepatitis rats, it is not yet possible to identify the mechanism(s) involved with any degree of certainty. Two major possibilities would appear to involve either (a) restoration of the structural integrity of the hepatocyte or (b) a direct biochemical effect upon the metabolic events disrupted in galactosamine-hepatitis. Since, however, normalization of biliary excretion occurs rapidly in our experimental hepatitis model in contrast to the lengthy period of clinical recovery from acute viral hepatitis, it appears probable that the observed effect on drug excretion is not dependent upon the longer term regeneration of hepatic tissue.

The possibility remains therefore that cyanidanol-3 may exert a direct effect on molecular events relating to biliary excretion. Watkins and Klassen /11/ have suggested that the reduced biliary excretion of drugs in galactosamine hepatitis may be brought about by an impairment in glucuronide conjugation, associated with reduction in the synthesis of UDP-glucuronic acid. Further research is however needed to establish a basis for possible cyanidanol involvement in restoring normal metabolic activity.

The ability of cyanidanol-3 to partially restore normal levels of biliary excretion and plasma clearance in the rat-galactosamine-hepatitis model suggest that concurrent therapeutic use of cyanidanol in man might reduce the toxic effect of certain drugs which would otherwise be contra-indicated in human liver disease.

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INVESTIGATION OF THE PROTECTIVE EFFECT OF (+)-CYANIDANOL-3 AGAINST PARACETAMOL-INDUCED MEMBRANE DAMAGE IN ERYTHROCYTES

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INTRODUCTION

The method of acid haemolysis has been used for a long time, mainly for the diagnosis of paroxysmal nocturnal haemoglobinuria /1/. Recently we elaborated a modified method according to which the haemolytic process, induced by hydrochloric acid, is performed at 37°C, with constant stirring in a Chronolog aggregometer. The gradual increase of the transmission of the erythrocyte suspension can be registered by a potentiometric recorder in the form of a dynamic haemolysis curve. By detecting changes at the time of full haemolysis, this procedure seems to be applicable for the investigation of the membrane-damaging and cytoprotective effect of different agents. Previously we gave an account of our examination /2/, carried out in order to clear up the membrane stabilizing effect of Catergen^R, (+)-cyanidanol-3, on human erythrocytes, under in vivo and in vitro conditions. According to our experiences, Catergen^R could provide certain protection against the haemolytic effect of hydrochloric acid.

Now we present the results of our examinations on the protective effect of (+)-cyanidanol-3 against paracetamol-induced membrane damage in mouse and human erythrocytes. According to Wendel /3/ the phase-I metabolism of the primary hepatotoxic paracetamol (acetaminophen) caused a dose-dependent lipid peroxidation in the mouse, which could be prevented, if the animals had been pretreated with liposomally entrapped reduced glutathion. The aim of our study was to investigate whether paracetamol-induced membrane damage can be demonstrated by shortening of the time of full haemolysis and whether this effect can be moderated or prevented by (+)-cyanidanol-3 pretreatment.

METHODS AND RESULTS

In our experiments the membrane-damaging effect of paracetamol was investigated in blood samples collected 2 hours following the oral administration of a 10 mg/kg dose of paracetamol to adult mice. At the same time also the determination of the SGOT and LDH levels was carried out. While the values of SGOT did not change significantly in comparison with the controls, the erythrocyte membrane damage could be well demonstrated by the decrease of the time of full haemolysis induced by hydrochloric acid - even after the administration of this, relatively small dose of the drug - as it can be seen in Fig. 1.

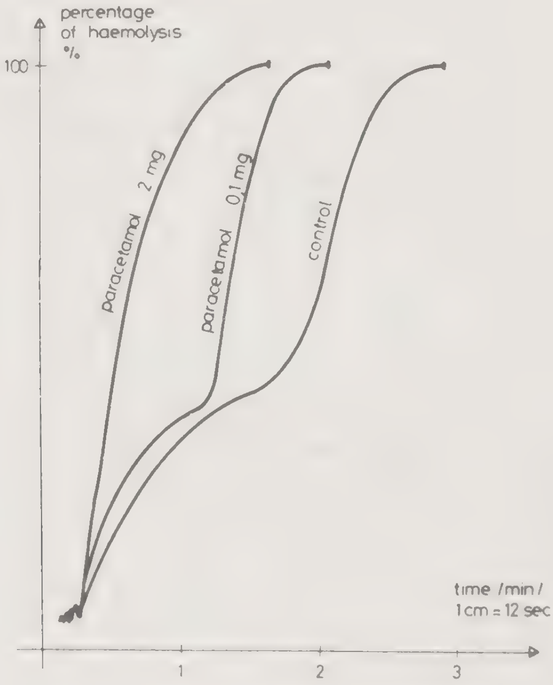

Fig 1 The effect of paracetamol on haemolysis in mice /in vivo/

Similar results were obtained when instead of paracetamol, the same dose of Scutamil-C, dissolved in distilled water, was administered. At the same time, the in vitro incubation of erythrocytes with paracetamol did not cause any alteration in the time of haemolysis, as compared with the controls. The investigation of the effect of (+)-cyanidanol-3 on the damaging effect of paracetamol was carried out in the following way. The animals were devided into 4 groups, each consisting of 6 mice. In the first and second, both drugs were added orally, while in the third and forth groups (+)-cyanidanol-3 was injected intraperitoneally, in a dose of 100 mg/kg body weight (Table 1).

Table 1.

Investigation of the protective effect of (+)-cyanidanol-3 against paracetamol induced membrane damage in mice.

time	form of administration			
0^h	C + P p.os p.os	C p.os	C + P i.p. p.os	C i.p.
2^h	E	P p.os	E	P p.os
4^h		E		E

C = (+)-cyanidanol-3

P = paracetamol

E = examination

The results are shown in Fig. 2. It was found that (+)-cyanidanol-3 can prevent the damaging effect of paracetamol, when injected parenterally two hours earlier; however if the two drugs were given simultaneously, no protective effect could be demonstrated.

In our human investigations 5 tablets of Scutamil-C - containing altogether 500 mg of paracetamol - were orally administered to 16 adult patients, without severe illness, and blood samples were collected with heparin at the beginning, then 2, 4 and 6 hours later. The same dose can be given for analgesia /4/. The samples were stored at 4°C, and the measurements performed usually within two hours. At the beginning and after 6 hours SGOT and LDH levels were also measured.

While in the serum levels of the different enzymes there were no changes, the time of full haemolysis decreased significantly after 4 hours, in 10 out of 16 patients. The results are shown in Fig. 3.

In 6 cases the paracetamol test was repeated following a 3 days Catergen^R pretreatment (3 x 500 mg/day). In these cases the above mentioned shortening of the time of full haemolysis, observed 4 hours after paracetamol intake, was markedly reduced, as it can be seen in Fig. 4.

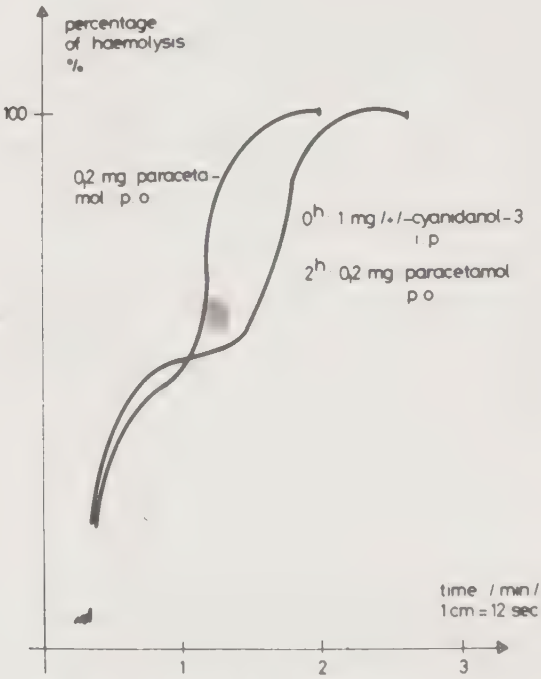

Fig. 2 The influence of /+/-cyanidanol-3 on the effect of paracetamol /in vivo/

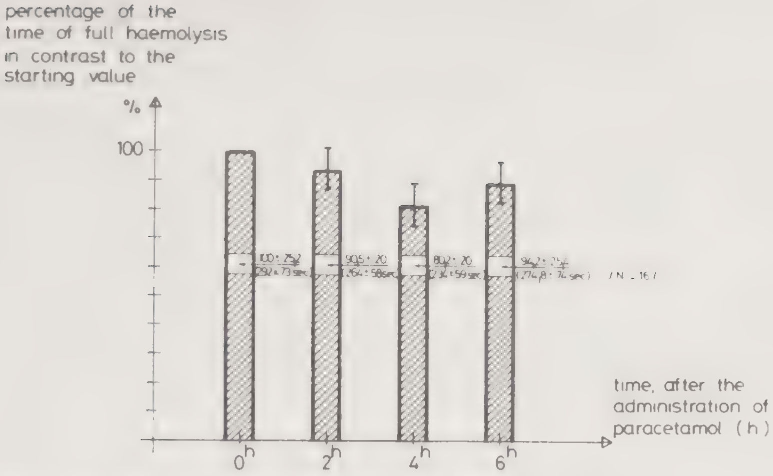

Fig 3 The effect of paracetamol / 500 mg = 5 tablets of Scutamil-C / on the time of full haemolysis / in vivo /

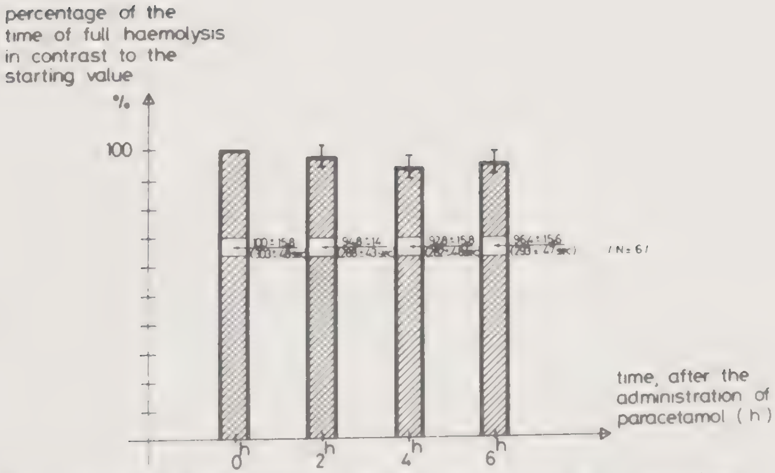

Fig 4 The effect of paracetamol / 500 mg / on the time of full haemolysis, following pretreatment with Catergen® / 3 x 500 mg / die, for 3 days /

DISCUSSION

The phenomenon of the decrease of time of full haemolysis after paracetamol ingestion can probably be explained by a certain damaging effect of the drug on the membrane of human erythrocytes, due to depletion of glutathione level and generation of free radicals (Fig. 5.), which cannot be completely eliminated by the glutathione peroxidase system, thus leading to lipid peroxidation in the erythrocyte membrane and subsequently to faster haemolysis under the effect of hydrochloric acid /5/.

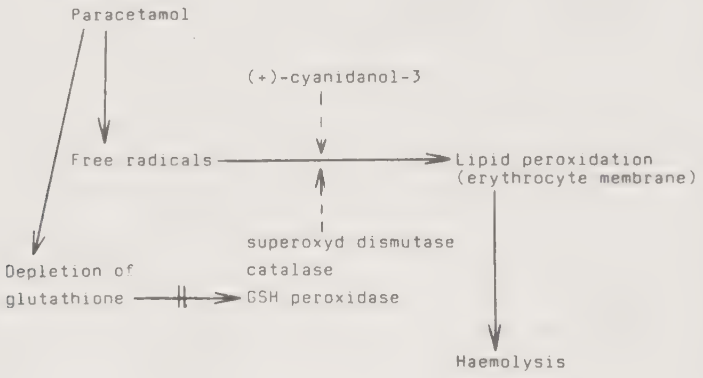

Fig. 5. Principle of the "paracetamol-test"

We found that, previously added Catergen^R, as a free radical scavenger, can provide certain protection for erythrocytes against the membrane-damaging effect of lipid peroxidation induced by paracetamol. This finding is similar to that of Kappus /6/ and is in accordance with the conclusion of Japanese authors /7/. On the other hand, our results make it reasonable to assume that the modified method of acid haemolysis can be used for the detection of moderate membrane damage, caused e.g., by lipid peroxidation.

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INTERACTION OF 3-O-METHYL-(+)-CATECHIN AND H_2-RECEPTOR BLOCKERS IN PIROXICAN- AND INDOMETHACIN-INDUCED GASTRIC ULCER MODELS OF RAT

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INTRODUCTION

As published by Parmar and Ghosh /1/ 3-O-methyl-(+)-catechin (3-OMC) has a marked anti-ulcerogenic effect in different experimental ulcer models.

The aim of this study was to investigate the possible interaction of the powerful anti-ulcerogenic H_2-receptor blockers cimetidine (CIM) and ranitidine (RAN) in combination with 3-OMC in the indomethacin- (IND) and piroxicam- (PXC) -induced gastric ulcer of the rat.

MATERIALS AND METHODS

The experiments were performed on female Wistar rats weighing 200-230 g. Prior to the investigations the animals were fasted for 24 hours, but allowed to consume water ad libitum.

IND-ulcer model

A single dose of 30 mg/kg of IND was given i.p. at the beginning of the experimental period (0 min.). In the 4th hour after IND administration the animals were killed and their stomach was removed /2/.

PXC-ulcer model

To establish the possible ulcerogenic effect of PXC the following pilot study was performed:

5, 10, 20 and 30 mg/kg PXC was given i.p. at the beginning of the experiment. Four hours later the animals were sacrificed and their stomach was removed.

The changes observed in the stomachs of the animals were evaluated in both ulcer models by Ulcer Index (U.I.) counting /3/.

Within each group the mean value ± SEM was calculated. The statistical analysis (*vs.* control group) was performed with the help of the Student *t*-test. Significant differences were assumed when the probability was less than 5%.

The drugs tested and their single i.p. doses in aqueous solution were:

3-OMC: 5 mg/kg;
CIM: 0.5 mg/kg; (Inj."Tagamet", S.K.and F., U.K.)
RAN: 0.5 mg/kg; ("Zantac", Glaxo, U.K.)

Treatments took place at 0 min and in the 2nd hour of the experimental period. The control animals received normal saline in the same volume as the drug solution.

RESULTS

The experimental results are presented in Tables 1 - 2 and 3.

Table 1

Effect of Piroxicam on the Gastric Mucosa of Rat

Group	Dose mg/kg i.p.	n	U.I. $\bar{x}$ ± SEM
PXC	5.0	5	3.5 ± 1.2
PXC	10.0	5	5.6 ± 1.8
PXC	20.0	5	6.2 ± 2.1
PXC	30.0	5	7.3 ± 2.4

In the main experiments 30 mg/kg PXC was used as the ulcerogenic agent.

Table 2

Interaction of 3-O-methyl-(+)-catechin and H_2-receptor Blockers in Piroxicam-induced Gastric Ulcer Model of Rat

Group	Dose mg/kg i.p.	n	U.I. $\bar{x} \pm$ SEM	U.I. Δ%
Control	---	10	7.3 ± 2.4	100.0
3-OMC	5.0	10	2.4 ± 0.9	32.9
CIM	0.5	10	2.6 ± 1.1	35.6
RAN	0.5	10	2.1 ± 0.8	28.8
3-OMC +	5.0			
CIM	0.5	10	4.2 ± 1.6	57.5
3-OMC +	5.0			
RAN	0.5	10	2.3 ± 0.8	31.5

Table 3

Interaction of 3-O-methyl-(+)-catechin and H_2-receptor Blockers in Indomethacin-induced Gastric Ulcer Model of Rat

Group	Dose mg/kg i.p.	n	U.I. $\bar{x} \pm$ SEM	U.I. Δ%
Control	---	10	16.8 ± 4.1	100.0
3-OMC	5.0	10	$4.7 \pm 1.4^{*}$	28.0
CIM	0.5	10	6.8 ± 1.9	40.5
RAN	0.5	10	$4.8 \pm 1.3^{*}$	28.6
3-OMC +	5.0			
CIM	0.5	10	9.5 ± 2.4	56.5
3-OMC +	5.0			
RAN	0.5	10	$4.9 \pm 1.6^{*}$	29.2

$* = P < 0.05$

DISCUSSION

According to Parmar and Ghosh /1/ 3-OMC, given alone, exerts a significant anti-ulcerogenic effect in experimentally induced IND-ulcer. In our experiments, simultaneous administration of CIM decreased this protective effect. No such interaction could be detected between 3-OMC and RAN. It seems therefore that this difference is due to the different chemical structures of the two H_2-receptor blockers rather than to a real receptorial antagonism.

PXC, similarly to IND, causes gastric ulceration in a dose-dependent manner in rat.

A similar interaction of 3-OMC and the examined H_2-receptor blockers was proved also in the case of PXC-induced ulcers.

SUMMARY

PXC, similarly to IND, causes gastric ulceration in a dose-dependent manner in rat.

It has been shown that CIM significantly lowers the anti-ulcerogenic effect of 3-OMC in either IND or PXC-induced gastric ulcer models, while RAN did not show any interaction with 3-OMC.

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SUBJECT INDEX

Achillea
  flavonoid aglycones in 213-220
Acifluorfen
  flavonoid formation induced by 189-192
2-Alkoxymethylchromones 55-60
Alkylresorcinols 23
Amanita phalloides 148
Amentoflavone 377
Americanin A 139, 146
Americanin D 31-37
Aminoflavan 40
Analgesics 15-29
Andrographis paniculata 312
Angiosperms 166
Anthocyanidins 273
Anthocyanin
  in Consolida orientalis 261-263
Anthocyanosides 293-301
  effect of
    on elastolytic activity 296
    on protein biosynthesis 295-297
Anthocyanosides effect of
    on the blood-brain barrier 297, 299
  therapeutic effects of 299-301
Antiarteriosclerotic agents 321-324
Antihepatotoxic activity
  of flavanolignans 146-152
Antilipoperoxidants 423-434
Antioxidants 423, 434
Antithyroidal drugs 418-421
Antiulcer activity 310-315
Antiviral activity
  of quercetin 339-348
    combined with interferons 333-338
Apigenin-7,4-di-O-methyl ether 312
Arachidonic acid 403-409, 425
Arteriosclerosis 321-324
Arylchromones 322-324
Ascorbic acid
  autoxidation of 428
Aurones
  as deiodinase inhibitors 415-421

- Barley 184-191
- Befunolol
  - ophthalmocological use of 304
- Benzofurans
  - cardiovascular effects of 306-307
- Benzopyranopyrazolines 47-52
- Benzopyrones
  - antiallergic effects of 307-309
  - antiulcer activity of 310-315
  - effects on the
    - cardiovascular system 303-306
    - central nervous system 303
    - gastrointestinal tract 309-315
    - inflammation 315-316
    - respiratory system 307-309
    - smooth muscles 304
    - vegetative nervous system 304
  - pharmacology of 303-317
- 3-Benzylidenechromanones 47-52
- 2-Benzylidene-1-tetralone 47
- 3-Benzylidene-1-thiochromanone 47-52
- Beverages
  - haze formation in 130, 135
- Bilary excretion 438
- Bilobalide 351
- Bioflavonoids 173
- Biosynthesis
  - of phylloquinone 210-211
  - of plastoquinone 209-210
  - of α-tocopherol 209-210
- Blood-brain barrier 353
- Blood platelets 373-378
  - adhesion 373-374
  - aggregation 374-377
  - secretion 374
- 3-Bromoflavanones 75
  - kinetics of dehydrobromination 75-83

- *Calcatrippae floss* 261
- Cannabinoids 15-29
  - analgesic activity of 27
  - synthesis of 15-29
- Capillary resistance
  - increase of 243, 246, 247
- Cardiovascular activity 279, 289
- Cardiovirus infections 340, 346
- Catechins 288, 292
- Catergen^{R} 445
  - protective effect of 445-450
- Cearonin 7
- *Centaurea phyllocephala* 179
- Chalcone derivatives
  - antiulcer activity of 313
- Chalcones 42
  - deiodinase inhibition by 414-419
- Chemotaxonomy 155-168
- Cherry peduncle 279-285
  - constituents of 279-281
  - in folk medicine 279
- Chicken leukocyte interferon 333
- Chloroplasts 195-211

Cimetidine 453-455
Cinnamic acid derivatives 415
^{13}C-NMR spectroscopy
  of benzopyranopyrazolines 51
Collagen
  role of, in diabetes 294-301
Compartmentation 195-211
Computer graphics 411, 413, 416-418
Conifers
  chemotaxonomy of 287
Consolida orientalis
  anthocyanin in 261-263
Co-oxidation 403-409
Cotinus coggygria 241-249
  polyphenols in 241-249
  therapeutical value of 241-249
Crataegus azarolus 172-176
(+)-Cyanidanol-3
  antiulcer activity of 310
  normalization of liver disease by 437-443
  protective effect of 445-450
Cyanidin
  isolation of 273-277
Cyclic AMP 375-378
Cyclodextrin 26
Cyclooxygenase 375-377, 423, 425-431, 433-434
  effects of flavonoids on 389-401
Cytoprotection 445

DAHP-synthase 195, 201, 202, 206
Dalbergia melanoxylon 2, 3
Dalcriodanin 1
Dehydrobromination 75
  mechanism of 75-83
Dehydroquinate
  hydrolase (DHQase) 195, 202, 206
  synthase (DHQ-synthase) 195, 202
Deiodinase
  inhibitors of 411-421
Delphinidin-3-rutinoside-5-glucoside 263
Desmethylbellidifolin glycosides 65
Diabetes 294-301
  effects of anthocyanosides in 294-301
Diabetic microangiopathy 293-301
m-Digallic acid 241, 245
Dihydrochalcones
  deiodinase inhibition by 414-415
2,3-Diphenylbenzo[b]furan 10
2,4-Diphenyl-5,6,7,8-tetrahydroquinoline 42
Distribution studies
  with *Ginkgo biloba* flavonoids 355-359
DNA synthesis
  stimulation of 370
Drug metabolism 437-443

Elastase 296-297
Elastin 294
Elimination reactions 75

Endothelial cells 373
Enzymes 195-211
  in barley and soybean 186-192
Ethylene carbonate
  hydroxyethylation with 103-111

Fabaceae 321
Flavanolignans 32-37, 139-152, 361-371
  stereochemistry of 142-146
  structure-activity relationship of 146-152
  synthesis of 146
Flavanone 40, 43
  reaction with
    ammonium acetate 43
    ammonium formate 40
Flavone
  from 3-bromoflavanones 75-76
Flavonoid aglycones
  as leaf exudates 155-168
  ecology of 167-168
  distribution of 166
  diversity of 162
  external 213-220
Flavonoid-O-glycosides 351-259
Flavonoids
  antiviral activity of 385-386
  antiviral effects of 339-348
  bioavailability of 353-357
  containing sulfur 85-91
  effect on natural killer-cells 325-330
Flavonoids
  effects of
    antilipoperoxidant 425, 428, 432
    antioxygen 425, 428, 432-433
    antiradicalar 424, 426, 431
    in enzyme systems 403-404, 407-409
    in vascular diseases 378
    on histamine release 382-384
    on human platelet function 373-378
    on mammalian cell systems 381-387
    on thyroid hormones 411-421
  enzyme influencing effects of 317
  enzyme inhibition by 385-387, 389-401
  in *Achillea* 213-220
  in *Cotinus coggygria* 241-249
  in extract of cherry stalk 279-285
  in folk medicine 171-179
  in Iraqi plants 171-179
  in *Mespilus germanica* 251-259
  in *Phyllocladus alpinus* 287-292
  in *Populi gemma* 223-231, 233-239
  in propolis 223-231, 233-239

Flavonoids
  in Prunus avium 279-285
  liver protecting activity of 148
  mode of action on platelets 376
  of Ginkgo biloba 351-359
  quantum chemistry of 93-100
  role of
    in lipoperoxidation 423-434
    in plants 183, 219
  structure-activity relationships 382-385, 415-420
  tissue-specific localization of 183-192
  tissue-specific production of 183-192
Folk remedies 171-179, 411, 418
Free radical formation 404, 409
Furochromones
  lipid-altering activity of 307

Gallic acid 241, 243, 245
Galloyl esters 116-118
Gastric ulceration 453-456
Ginkgo biloba 351-359, 423
  extract of leaves 351-359
    biological effects of 353
    components of 351-353
    distribution studies with 355-359
    kinetic studies with 355-359
    metabolic studies with 355-359
Ginkgolides 351
Glycine max. 184
Glycogallic compounds 243
Goitrogens 413, 414
Gossypin
  analgesic activity of 303
Greveichromenol 60

Haemolysis 445-450
  dynamic curve of 445
Helleboraceae 261
Hepatitis
  induced by galactosamine 437-443
Hepatocytes 412, 416, 418
Herpes simplex virus type-1 (HSV-1) 333-338
Heteroarylchromones 323
Hexahydroxydiphenoyl esters 116-118
Histamin release
  effects of flavonoids on 382-384
^{1}H-NMR spectroscopy
  of benzopyranopyrazolines 50
Hordeum vulgare 184
Horseradish peroxidase (HRP) 403-409
H_2-receptor blockers 453-456
  interaction with 3-O-methyl (+)catechin 453-456
Human leukocyte interferon (HuIFN-α) 333
Human natural killer (NK) cells 325-330
Human platelets 389-401

Hydnocarpin 142-143
Hydroperoxyeicosatetraenoic acids (HPTE) 403-409
2′-Hydroxychalcone 42
Hydroxyetylation 103-111
  of quercetin 107
Hydroxyethylrutosides 374, 377
Hydroxymethylchromones 60
2-(2′-Hydroxyphenyl)-4,5-diphenyl-5H[1]-benzothiapyrano-[4,3-b]-pyridine 44
  -S-1,1-dioxide 44
Hyperlipidemia 322
Hypertensive activity 289-292
Hypolaetin-8-glucoside 314, 316

Imipramine 437, 439, 441
Immunocompetent cells 346
Immunodeficiency 346
Inclusion complexes 26
Indomethacin 453-456
Inhibitors
  of deiodinase 411-421
Interferon 325-326, 328, 330, 333-338, 345
Iodothyronine deiodinase (ITH-D)
  inhibitors of 411-421
Iodothyronines 411-412
Iraqi plants
  flavonoids in 171-179
  pharmacological screening of 171-179
Isoflavones
  heteroanalogues of 323
  hypocholesterinemia effect of 322-324
Isoflavones
  hypolipidemic effect of 322-324
  in treatment of arteriosclerosis 321-324
  syntheses of 322-323
Isosilybin 142-147
Isosilychristin 140-144

K-562 cell line 327
Kaempferol
  determination of, in blood and urine 355
  glycosides 265-269
    in soybean 184, 187-188, 191
3-O-β-D-xylopyranosyl(1-2)-β-D-glucopyranoside 265-267, 269
  ^{13}C-NMR data of 268
  isolation of 265-269
  structure elucidation of 265-269
Kinetic studies 75-83

Latinone 2, 3-6
Leaf exudates 155-168
Leguminosae 2
Leuckart-Wallach reaction 40
Lilium candidum L.
  flavonoids in 165-169
  in domestic medicine 265
  perianth of 265-267, 269
  stamen of 265-267, 269
Lilyn 265, 267-269
Lipid peroxidation 445, 450

Lipoperoxidation
  role of flavonoids in
  423-434
Lipophilic compounds 161, 168
Lipoxygenase
  effects of flavonoids on
  389-401
Lithospermum off. 411, 413-414
Liver disease 361, 371, 437
Liver microsomes 425

Macrophages 346
Mammalian cell systems 381-387
Medicinal plants 412
Medlar 251-259
Mellatinone 2, 3
Membrane damage 450
  protection against 445-450
Mespilus germanica 251-259
  flavonoids in 251-259
3-Mesyloxyflavanones 85-91
  reactions with S-nucleophiles 85-91
3-O-Methyl-(+)-cathechin
  effect of, in experimental gastric ulcer 453-456
  interaction with H_2-receptor blockers 453-456
  pharmacological effect of 311-312
7-O-Methyllatinone
  synthesis of 6
Mice
  antiviral effects of flavonoids in 339-348
Morin 339-340
Myricetin 243, 244-245
Myrtus cummunis 177-178

Nabilol 18
  analogues of 18
Naringenin 314
Neoflavonoids 1-13
Neosilyhermin A 140-143
Neosilyhermin B 140-143
Nepeta hindostana 315
Nepitrin
  anti-inflammatory action of
  315
Novicardin 279-285

Oxidative phenol coupling 33

Paracetamol 445-450
  effect of, on haemolysis
  446
Peroxidatic drug activation
403-409
Phase-transfer reactions
85-88
Phenanthra-1,4-quinones 1-13
  biological activity of 13
Phenylbenzo[b]furans 7-10
Phenytoin 437, 439-442
Phosphodiesterase (PDE)
376-378
Phospholipase 386
Phylloclaudus alpinus 287-292
Phylloflavan 287-292
  mechanism of activation of
  190-292
  pharmacology of 289-292
  structure of 288

Phylloquinone
  biosynthesis of 210-211
Phytochemical identification 174-175
Phytochemical standardization 174
Phytolacca americana 146
Piroxicam 453-456
Plant
  ecology 213-220
  epidermis 183-187, 190-191
  herbivore interactions 126-128
  mesophyll cells 183-192
  metabolites 115
  polyphenols 113-137
  taxonomy 213-220
  tissue types 183
  vascular strands 186
Plastoquinone 195, 196
  biosynthesis of 210-211
Polyphenol complexation 113-137
Populi gemma 223-231
  components of 223, 233-239
  GC/MS analysis of 223-231
Populus nigra 234
Prenylquinones 196, 208-209
Proanthocyanidins 116, 136-137, 176
Procyanidin
  in Cotinus coggygria
    antiviral effect of 243, 248
    curative effects of 244, 249
    effects in clinical cases 244, 249
Procyanidin
  in Cotinus coggygria
    microbiological effects of 244, 249
Procyanidins 130-137, 172-176
Propolis 223-231, 233-239
  components of 223-234, 233-239
    pharmacological actions 234
  GC/MS analysis of 223-231
  Hungarian 233-239
Propylene carbonate
  hydroxyethylation with 107-109
Prosopis farcta 176
Protein
  biosynthesis 295
  kinase 386
  synthesis 369-370
Proteins
  complexation
    with carbohydrates 122-123, 137
    with polyphenols 115, 128, 136
  irreversible association with polyphenols 128-137
Protoplasts 183-192
Prunus avium 279
Prunus cerasus 279
Ptaerochromenol 60
  methyl ether 60
Puerarin
  cardiovascular action of 306
Pyranochromones 55-61

Quantum chemistry 93-100
Quercetin 382-387
  antiviral effect of 339-348
    in combination with interferons 333-338
  hydroxyethylated derivatives of 107-109
  immunological effect of 339-348
  inhibition of human NK cytotoxicity by 325-330
  interaction with interferon 330
  toxicity of
    in mice 347
Quercetin-3,7-diglycosides 254-256

Radix pueraria 405
Rantidine 453-455
Raspberry 275
Rat liver microsomes 412, 418
RNA synthesis
  stimulation of 361-369
Rosaceae 251
Rubus ideus 275

Santolinoidea 216-217
Scutamil-C 447
Secondary metabolites of plants
  as deiodinase inhibitors 413-420
Shikimate
  kinase (SK) 195, 196, 204-206
  oxidoreductase (SORase) 195, 196, 202, 206
Shikimate
  pathway 195-211
    enzymes of 196-211
Sidiretis mugronensis 314, 316
Silandrin 139, 141-143
Silibinin 361-367
  effects of
    in liver cells 361-371
    on gene transcription 370-371
    on nucleic acid synthesis 361-371
    on protein synthesis 368
Silybin 139-152
Silybum marianum 139-152, 361-371
Silychristin 139-140, 143-144
Silydianin 139-140, 142-143
Silyhermin 140-142
Silymarin 139-152
Silymonin 139, 141-143
S-Nucleophiles 85-91
Sophoradin 313
Sophoranone 313
Sophora subprostata 313
Soybean 184-191
Spinach 196, 197, 200-210
Structure-activity relationships 323-324, 375
  in iodinase inhibitors 411, 415-421
Synthesis
  of Americanin D 31-37
  of benzopyranopyrazolines 47-52
  of cannabinoids 15-29
  of neoflavonoids 1-13
  of pyranochromones 55-61

Tannins 177, 116-118
Terpenoids 161, 166-167
1-Thiaflavanone 44
  1,1-dioxide 44
Thioflavanones 85-91
Thrombosis 373, 374, 378
Thymus vulgaris 304
  spasmolytic effect of
  flavonoids in 305
Thyroid hormones (T_4, T_3, rT_3, $3,3'$-T_2) 411-421
α-Tocopherol
  biosynthesis of 209-210
Total-ion chromatograms
  of *Populi gemma* components
  223-231
  of propolis components
  223-231
Trifolium protense 322

UV spectra
  calculated
    of aurone 99
    of chalcone 100
    of chromone 96
    of flavone 98
    of isoflavone 98

Vaccinum myrtillus
  anthocyanosides in 293-361
  in treatment of diabetic
  microangiopathy 1-9
  pharmacological properties
  of 1-9
Venoruton 243, 245, 246
Virus
  Herpes 339
  Mengo 339-348
  Sindbis 339-341

Xanthone glycosides
  synthesis of 63-72
Xanthones 63-72

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The reputation of the periodically organized Hungarian Bioflavonoid Symposia is reflected in the wide international attendance at these meetings.

The Seventh Symposium, held in Szeged, Hungary, May 16–18, 1985, attracted participants from 15 countries. They contributed or listened to lectures dealing with all aspects of flavonoid research. Such research involves the fields of chemistry, biochemistry, phytochemistry, plant physiology and taxonomy, pharmacology and medical practice.

This broad interdisciplinary approach is found in the present volume, which comprises 44 papers. The recent results from the various fields and their connections with one another will make the book interesting to every scientist whose work is in any way connected with the research or use of flavonoids and related compounds.

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